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THE EFFECT OF pH AND SALT CONCENTRATION ON AQUEOUS EXTRACTION OF BRAIN PROTEINS AND LIPOPROTEINS*

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INTRODUCTION

A NUMBER of papers have recently appeared describing studies on various types of aqueous extracts of brain proteins, particularly with relation to fractionation of such extracts by electrophoresis. In these studies, the rate of extraction of proteins by physiological saline has been investigated (NAKAMURA, HAYASHI and TANAKA, 1954; HAYASHI, 1953) and the effect of the pH of the extracting solution on the subsequent electrophoretic patterns has been determined (KEUP, 1955; POLYAKOVA and GOTOVTSEVA, 1957). However, to our knowledge, no systematic study has been undertaken to determine the conditions necessary for extraction of the maximum amount of protein material from brain in aqueous medium. In connexion with our work on brain proteins and lipoproteins, these data were requisite for the development of satisfactory fractionation methods, so a survey of the effect of pH and ionic strength on the amount of material extracted in aqueous solution was carried out. To this end, we prepared extracts at pH's from 4 to 11·6 and ionic strengths from 0 to 5 and determined the amounts of nitrogen and phosphorus which could be precipitated by trichloracetic acid from these extracts. The results of these experiments are reported in this paper as well as some chemical analyses and the results of fractionation studies on the extracts thus obtained. A preliminary report of this work has been published previously (LEBARON and FOLCH, 1958).

The main reason why these data had not been obtained previously for brain tissue is that the high proportion and variety of lipids found in this tissue are bound to each other and to proteins in various types of complexes which easily form emulsions and suspensions in aqueous media that are difficult to resolve. We have found that these mixtures can be separated by the use of a relatively high centrifugal force, and water-clear extracts can be thus obtained. Using the method detailed below, maximum extraction occurs when the extracting solution has an ionic strength of from 2·5 to 3 and when the pH of the homogenate is between 6 and 9. The pH of homogenates made with unbuffered salt solutions is within the optimum range. About 20 per cent of white matter protein and 50 per cent of grey matter protein can thus be obtained in aqueous solution. Such extracts have been separated by dialysis into globulin and albumin fractions, the former containing about 25 per cent lipid, of which about one-fifth is cholesterol.

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EXPERIMENTAL

Analytical methods. The analytical methods used in this work have been described in previous papers from this laboratory (LEBARON and FOLCH, 1956) except that cholesterol was determined by a modification of the procedure of KINGSLEY and SCHAFFERT (1949).

General method of extraction. The general procedure for preparation of aqueous homogenates from brain was developed in collaboration with other members of the laboratory staff, particularly Dr. PORTER (PORTER and FOLCH, 1957).

Beef brains were obtained fresh from the slaughter house and extracted within a few hours of the death of the animal. They were first washed, stripped of meninges and chilled and then grey or white matter was dissected out. This tissue was then weighed and ground as it froze at −10°C with a mortar and pestle previously chilled to −10°. After the tissue had become too stiff to work, it was allowed to freeze to −10° while covered with aluminium foil to prevent drying. It was then removed to a 4° cold room and ground again while thawing as the appropriate salt solution, previously chilled to 4° was added in small portions. This extracting medium was added in the proportion of 10 ml/g fresh tissue. The resulting homogenate was then stirred mechanically for 15 min at 4° and centrifuged at 0° for 30 min at 40,000 *g* in a Spinco preparative ultracentrifuge. The water-clear supernatant or internatant extracts were carefully removed by decantation or pipetting.

Effects of variations in pH *and ionic strength on amounts of material extracted by aqueous salt solutions.* Using the procedure described above, extractions were carried out using the solutions listed in Table 1. Aliquots (10 ml) from the resulting extracts

TABLE 1.—COMPOSITION OF SALT SOLUTIONS USED FOR EXTRACTION OF PROTEINS AND LIPOPROTEINS FROM BRAIN

Salt used	Buffer used*	Final ionic strength	Homogenate pH†
NaCl	—	0·04, 0·09, 0·17, 0·2, 0·34, 0·43, 0·86, 1·0, 1·28, 1·71, 2·5, 3·85, 4·0, 5·0, 5·13	6·0–6·2
NaCl	HAc–NaAc	3·0	4·1, 4·2, 4·4, 4·5, 4·8, 5·1, 5·3, 5·4
NaCl or KCl	K_2HPO_4–KH_2PO_4	3·0	5·1, 5·4, 5·7, 5·9, 6·0, 6·1, 6·2, 6·4, 6·6, 7·1
NaCl	Glycine–NaOH	3·0	9·0, 9·3, 9·7, 10·1, 10·4, 11·0, 11·6

* Buffers were 0·1 M. The rest of the ionic strength was made up with NaCl and/or KCl.
† pH's were measured at 4° using a Beckman model H2 pH meter.

were treated with 3 ml of 20% TCA and the resulting precipitates collected by centrifugation and washed with 5 ml of 5% TCA. These residues were then dissolved either in hot 2 N-NaOH and analysed for total nitrogen, or in warm concentrated HCl and analysed for total phosphorus. The resulting data for TCA-precipitable nitrogen and phosphorus are given in Figs. 1–4. It can be seen that the conditions for maximum extraction from both grey and white matter occur at an ionic strength of around 2·5–3 and that there is little increase in extraction above pH 6 until rather strong alkaline solutions are used. It should be noted that a number of different anions and cations were included in the solutions used but, when the pH and ionic strength were kept constant, there was no variation in the amount or nature of material extracted which could be attributed to the change in the nature of the ions present.

Completeness of extraction. To determine if the material extracted under the conditions of maximum extraction was an exhaustible fraction and whether or not it included all materials which would be extracted at lower ionic strengths, two types of experiments were carried out: (a) re-extraction with solutions of the same salt concentration, and (b) re-extraction with water, and solutions of lower salt concentration.

(*a*) *Repeated extraction with* 20% NaCl. Extractions were carried out as outlined above using unbuffered 20% NaCl as the extracting medium, i.e. conditions for maximum extraction (v.s.), and the resulting volumes of residue and supernatant noted. The residues were then repeatedly re-extracted with 20% NaCl by suspending, stirring and centrifuging as before, all volumes again being noted. TCA-precipitable nitrogen was measured in each successive extract and, in each case, the amount found corresponded closely to the amount to be expected from dilution of the portion of the previous extract left wetting the residue. This indicated that the fraction being extracted was exhaustible.

(*b*) *Attempted re-extraction of the residue at lower ionic strengths.* Brain tissue was extracted with 20% NaCl by the procedure described above and the residue washed by re-suspending it in a volume of water equal to that of the original supernatant and re-centrifuging at 40,000 **g**. This was repeated as many as four times. In parallel experiments, very dilute HCl or NaCl solutions, varying in pH from 4·35 to 6·1, were used for further washings after only two washings with water. In all cases, the supernatants were tested for protein by treating with 20% TCA as described above. Apart from the expected washing out of the proteins extractable with the 20% NaCl, no soluble proteins were obtained. In some cases the supernatants were cloudy, due to resuspension of very light solids during braking of the centrifuge or decanting. In these cases, the cloudiness could be eliminated by recentrifuging the decanted supernatant at the same gravitational force or by pipetting aliquots from the tubes without decantation. It was therefore concluded that, under the conditions described, no water-soluble proteins were extracted at very low ionic strength after a preliminary extraction with 20% aqueous NaCl.

Fractionation and analyses of 20% NaCl *extracts.* The fraction obtained by the procedure described above under conditions of maximum extraction is obviously a mixture of various proteins and lipoproteins. The fractionation and analyses of these mixtures have been started. Extracts obtained from fresh beef brain grey or white matter using 20% NaCl as the extracting solution were dialysed exhaustively at 4° against distilled water. A bulky globulin precipitate formed, and this was separated by centrifugation. The residue was re-suspended in distilled water and both this suspension and the original supernatant (albumin fraction) were lyophilized. The results of analyses of these fractions are given in Table 2.

In addition, the two fractions from grey matter and the globulin fraction from white matter contain nucleic acids, although the exact values are not yet available because of the inadequacy of the method of estimation.

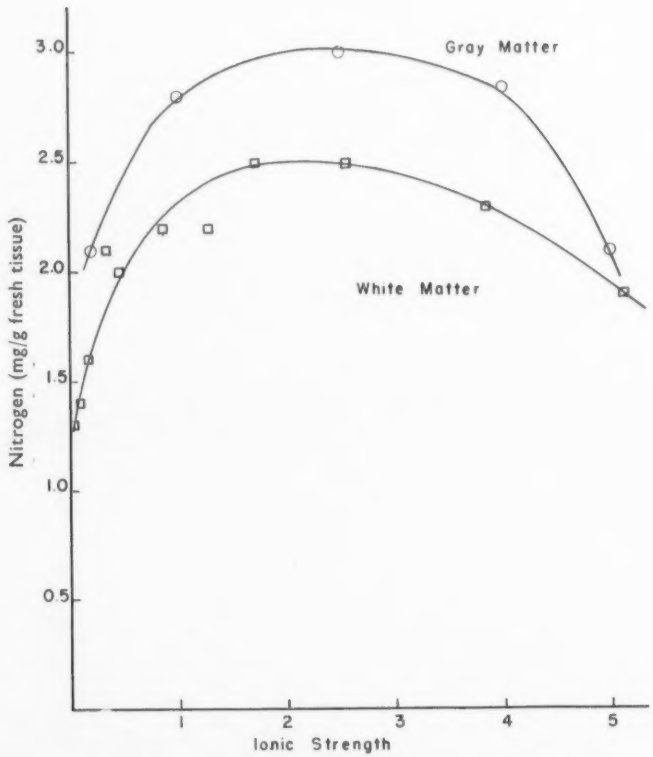

FIG. 1. Amounts of N precipitable by trichloroacetic acid from extracts obtained from fresh beef brain grey (○) and white (□) matter using unbuffered solutions of varying ionic strength whose compositions are given in Table 1.

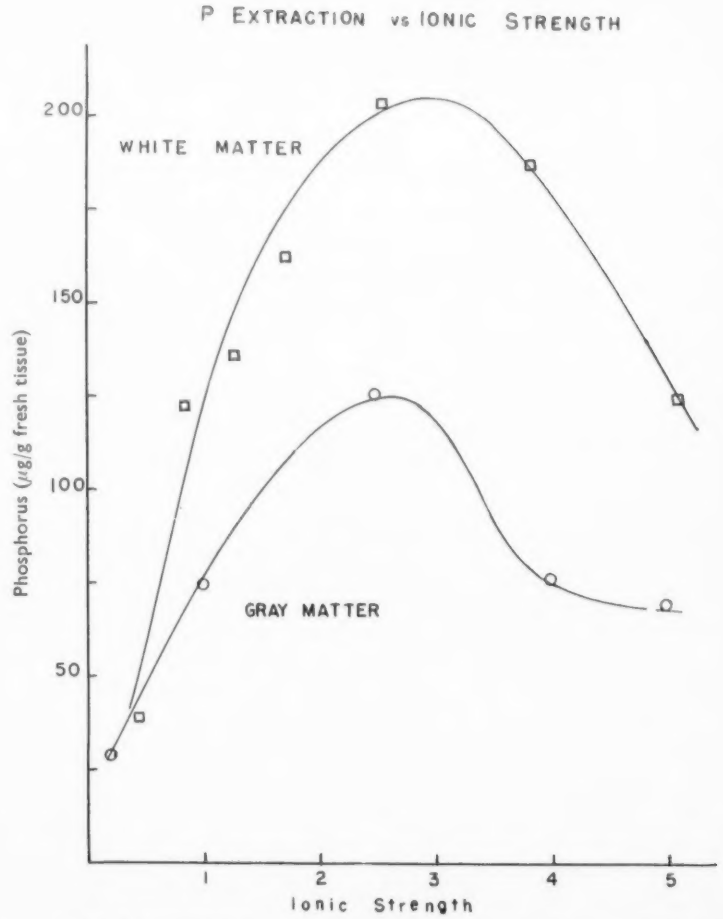

FIG. 2. Amounts of P precipitable by trichloroacetic acid from extracts obtained from fresh beef brain grey (○) and white (□) matter using unbuffered solutions of varying ionic strength whose compositions are given in Table 1.

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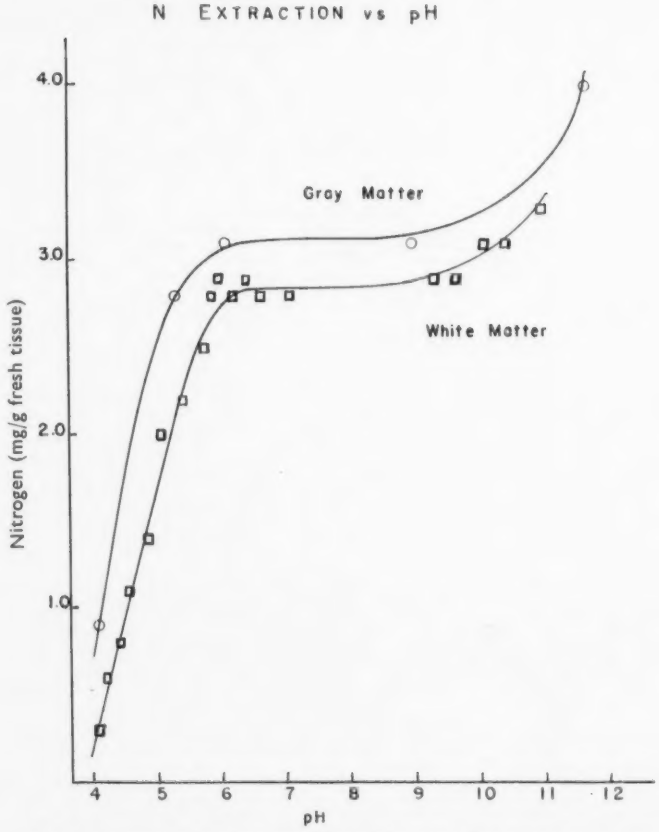

FIG. 3. Amounts of N precipitable by trichloroacetic acid from extracts obtained from fresh beef brain grey (○) and white (□) matter using solutions of ionic strength 3·0 and varying pH's whose compositions are given in Table 1.

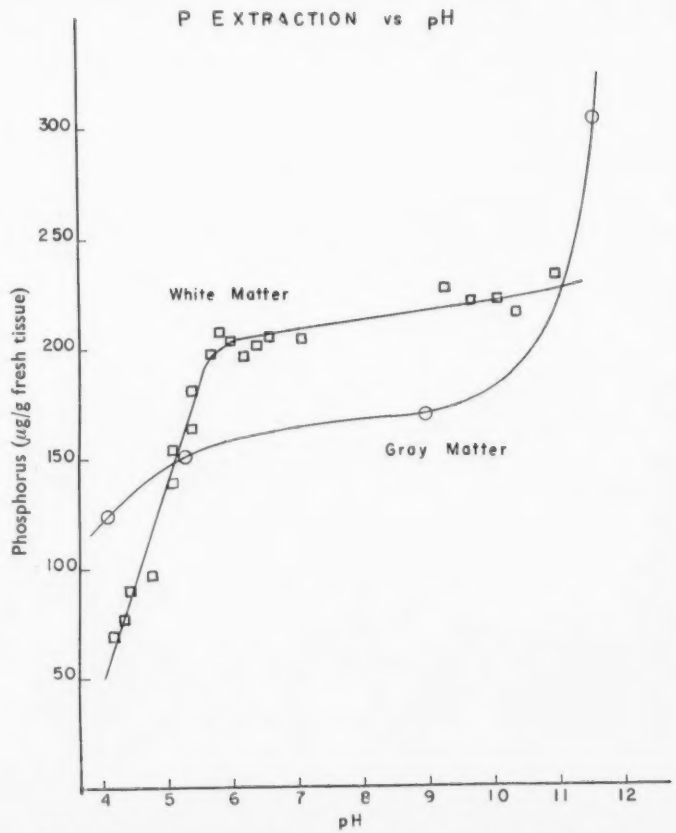

FIG. 4. Amounts of P precipitable by trichloroacetic acid from extracts obtained from fresh beef brain grey (○) and white (□) matter using solutions of ionic strength 3·0 and varying pH's whose compositions are given in Table 1.

TABLE 2.—ANALYSES OF FRACTIONS OBTAINED BY DIALYSIS OF 20% NaCl EXTRACTS OF GREY AND WHITE MATTER

	Grey matter		White matter	
	Albumin*	Globulin*	Albumin*	Globulin*
Weight (mg/g fresh tissue) ..	3·8	34·0	2·6	14·0
Total P (%)	0·15	0·80	0·48	1·04
Total N (%)	16	11	15	11
Extractable lipid† (% of fraction wt.)		22·0		28·7
Lipid-P (% of lipid wt.) ..		2·27		1·92
Cholesterol (% of lipid wt.) ..		20·9		24·4
Lipid galactose (% of lipid wt.)		8·5		8·6

* Albumin fractions are soluble, undialysable materials after exhaustive dialysis against distilled water at 4°. Globulin fractions are insoluble, undialysable substances after the same treatment.

† Solids extractable with $CHCl_3$:CH_3OH 2 : 1 (v/v).

The lyophilized albumin fractions from either grey or white matter can be completely dissolved in water and give a solution of pH 4–5. When this pH is altered by addition of dilute acid or alkali through the range of pH 3–11, a reversible precipitation occurs in the range of pH 5·5–6·5. The precipitate thus obtained amounts to 20 per cent of the total albumin nitrogen in the case of white matter and 26 per cent in the case of grey matter.

DISCUSSION

The purpose of the work described in this paper was to define the conditions for extracting the maximum amount of water-soluble protein from brain tissue as a first step in a scheme for fractionation of brain proteins. Further steps in this proposed scheme are still being worked out, but the basic pattern is to obtain water-soluble proteins as above, and then to obtain proteolipids (FOLCH and LEES, 1951) by extraction of the insoluble residue with 2 : 1 (v/v) $CHCl_3$: CH_3OH. Subsequent extraction of the $CHCl_3$:CH_3OH-insoluble residue may then yield other fractions such as phosphatidopeptides (LEBARON and ROTHLEDER, 1958). Preliminary experiments have indicated that the major portion of the proteolipids does remain after aqueous extraction and can still be extracted with $CHCl_3$:CH_3OH.

A second aim of the work described above has been to preserve as nearly as possible the 'native' state of the proteins and their complexes with lipids. For this reason, the procedures are not strictly analagous to others reported previously, and comparison of yields obtained by the various procedures is difficult. It is also made particularly difficult by the lack of a generally accepted value for the percentage of protein in brain. The analyses reported in the literature have varied greatly (FOLCH and LEBARON, 1957), depending upon the methods used and the source of the material. A complete discussion of this problem is beyond the scope of the present paper. However, if one uses average figures of 8·0 g/100 g for total protein in grey matter and 8·1 g/100 g in white matter, one can calculate that 47 per cent of grey matter protein and 21 per cent of white matter protein are extracted by the procedure described above. For comparison, PALLADIN and GORYUKHINA (1947), by successive extractions of grey matter

with water, 4·5% KCl and 0·1 N-NaOH, obtained 31·0 per cent, 28·3 per cent and 36·3 per cent of the proteins respectively, and in similar experiments with white matter 19·6 per cent, 23·6 per cent and 34·7 per cent respectively. By calculating on the assumption that 10 per cent of the fresh weight of the tissue is protein, the following values have been reported for whole brain: 12·5 per cent of total protein extracted at pH 8·1 (KEUP, 1955); 48–56 per cent of total protein extracted in 0·1 M-veronal-acetate buffer at pH 8·6 (HOFMANN and SCHINKO, 1956); 'about 50 per cent' of total protein extracted in 0·05 M-barbitone-citrate buffer at pH 8·6 (ROBERTSON, 1957). Our results would tend to confirm the conclusion of POLYAKOVA and GOTOVTSEVA (1957) who found that extraction with unbuffered salt solution provided conditions of optimum pH as judged by the results obtained on subsequent electrophoresis.

Because the fractions described here are mixtures, the preliminary studies on their chemical composition are of limited value. However, the finding of substantial amounts of cholesterol bound to water-soluble brain proteins, in confirmation of earlier work of KONNIKOVA (1950), is of interest. FINEAN (1957) has reported that the cholesterol of myelin is associated with proteins, at least in a very weak linkage. But the cholesterol isolated by the procedure detailed in this paper is probably not a constituent of myelin since more was obtained from grey matter than from white matter. In fact, the rather similar overall composition of the fractions obtained from both grey and white matter and the smaller yields obtained from the latter tissue would seem to indicate that at least the major part of the fraction extracted by this method is composed of intracellular lipoproteins and proteins rather than myelin constituents. A substantial amount of the material isolated from white matter may thus be of glial rather than neural origin.

SUMMARY

A technique is described for the preparation of reproducible, exhaustible aqueous extracts of proteins and lipoproteins from fresh brain tissue. With this technique, extracts were prepared with unbuffered salt solutions of ionic strengths from 0 to 5·1 and with solutions having pH's from 4 to 11 and an ionic strength of 3·0. Maximum amounts of TCA-precipitable nitrogen and phosphorus were obtained at an ionic strength of about 3 and pH values of from 6 to 9. Dialysis of extracts made from bovine grey or white matter with unbuffered 3 M-NaCl yielded lipid-free albumin fractions and globulin fractions containing about 25 per cent lipid. About 25 per cent of this lipid was cholesterol.

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A SIMPLE PROCEDURE FOR THE PREPARATION OF BRAIN SULPHATIDES*

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INTRODUCTION

THE existence of brain lipides which contain sulphur, i.e. sulphatides, has been recognized since the time of THUDICHUM (1901). However, because of the difficulties encountered in their isolation, only one sulphatide has ever been isolated and characterized to any extent, i.e. the potassium salt of cerebron sulphuric acid prepared by BLIX (1933). BLIX found equimolar amounts of cerebronic acid, sphingosine, and galactose in hydrolysates of his compound. Since these were the same constituents as had been found in cerebrosides, he concluded that his compound had the structure of a cerebroside esterified with sulphuric acid (Fig. 1). THANNHAUSER, FELLIG and SCHMIDT (1955), using THANNHAUSER and BONCODDO's method of preparation (1953), have shown that the esterification of the sulphuric acid occurs

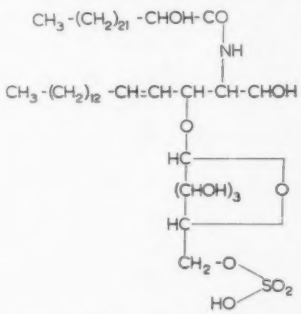

FIG. 1. Cerebron sulphuric acid.

on the primary hydroxyl group of carbon-6 of the galactose moiety. Both the procedure of BLIX and that of THANNHAUSER are lengthy and involved, and the amounts of sulphatides obtained correspond to less than one-tenth of the sulphur in fresh white matter. Although this low yield may be the result of factors inherent in the method, the possibility must be kept in mind that the bulk of the sulphatides may not exist in the form of cerebron sulphuric acid and that this sulphatide may represent a quantitatively small fraction which can be isolated relatively easily. Recently, RADIN, MARTIN and BROWN (1957), studying the metabolism of galactolipides in rat brain, have separated a sulphatide fraction from total lipides after their passage through Florisil and mixed ion exchange resins. No attempt was made to characterize

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the fraction obtained. In the course of studies on bovine brain lipide sulphur in this laboratory, a new group of lipoproteins designated as proteolipides, and having solubility properties akin to those of the lipides, was isolated (FOLCH and LEES, 1951). About half of the sulphur in brain total lipide extracts prepared according to FOLCH *et al.* (1951*a*) is present, not as sulphatide sulphur, but as part of the sulphur-containing amino acids of the protein moiety of the proteolipides.

This paper presents a rapid method for the isolation of sulphatides from brain white matter by a mild procedure and in good yield. The two main fractions which are obtained account for half of the sulphatide sulphur of the starting material. The procedure, which is based on the distribution of lipides in a series of related two-phase systems and is referred to as the method of 'linked distributions', consists of distributing the lipides between the two phases of a solvent system in which the sulphatides have a distribution coefficient markedly in favour of one of the phases, collecting that phase, adding to it a solvent or combination of solvents to make a second two-phase system, and continuing this type of procedure until the sulphatides are adequately concentrated. From several hundred grams of brain white matter, half of the sulphatides of the starting material can be obtained in two main fractions in 2 days. One fraction is phosphorus-free; the other contains both phosphorus and sulphur. The analysis of the former fraction suggests that it is an essentially pure sulphatide which is probably similar to BLIX's cerebron sulphuric acid. Although the complete proof of its structure is still lacking, it has been found that (a) its elementary composition is the same as that of cerebron sulphuric acid, (b) the sulphur exists as sulphuric acid esterfied with the organic portion of the molecule, and (c) all of the carbohydrate is present as galactose. The second fraction obtained is a lipide mixture which can be separated into a phosphatide and a sulphatide fraction by passage through a Florisil column. The sulphatide fraction thus obtained appears to be a mixture of cerebron sulphuric acid and one or more phosphorus-free lipides.

EXPERIMENTAL

Analytical Methods

Phosphorus was estimated by a modification of the method of SPERRY (1942); galactose by a modification of the method of TILLMANS and PHILIPPI (1929); nitrogen was estimated by wet digestion with H_2SO_4, K_2SO_4 and $CuSO_4$, followed by nesslerization of the digest; α-amino acid nitrogen by its reaction with ninhydrin in the procedure of VAN SLYKE, DILLON, MACFADYEN and HAMILTON (1941); total amino nitrogen by the nitrous acid method of VAN SLYKE (1929). Acetalphosphatides were estimated by the method of FEULGEN and GRÜNBERG (1939) as modified by Dr. G. SCHMIDT (unpub.); cholesterol was estimated by a modified Liebermann–Burchard reaction (KINGSLEY and SCHAFFERT, 1949); reducing sugar by the MILLER and VAN SLYKE procedure (1936). Sulphur was estimated as benzidine sulphate after oxidation with aqua regia (LEES and FOLCH, in press).

Reagents

Chloroform, analytical reagent.

Methanol, analytical reagent.

Chloroform : methanol (2 : 1 v/v)

Petroleum ether, analytical reagent, b.p. 30–60°. The petroleum ether is re-purified by shaking it with about one-eighth its volume of concentrated sulphuric acid and letting it stand in contact with the acid for several days with frequent shaking. The darkened sulphuric acid is replaced with fresh acid and the procedure repeated until no darkening of the acid occurs after contact with petroleum ether for several days. The petroleum ether is then washed repeatedly with water until the pH of the washings remains constant; it is then washed once with 10% Na_2CO_3, several times with water and

finally distilled over a boiling-water bath, the first sixth and the last tenth of the distillate being discarded.

N-KCl.

Florisil (activated $MgSiO_3$, 60–100 mesh obtained from the Floridin Company, Talahassee, Florida). It is washed by shaking with 5 volumes of chloroform : methanol mixture and filtered; the washing and filtering are repeated twice and the final residue is dried in an oven at 50°.

Isolation Procedure

'*Linked distributions*' (Fig. 2)

While the procedure has been carried out consistently at 22°C in our laboratory, it would appear that such strict temperature control is not necessary and that room temperature, with avoidance of excessively high summer heat, is adequate.

Step 1. A washed total lipide extract is prepared as described by FOLCH, LEES and

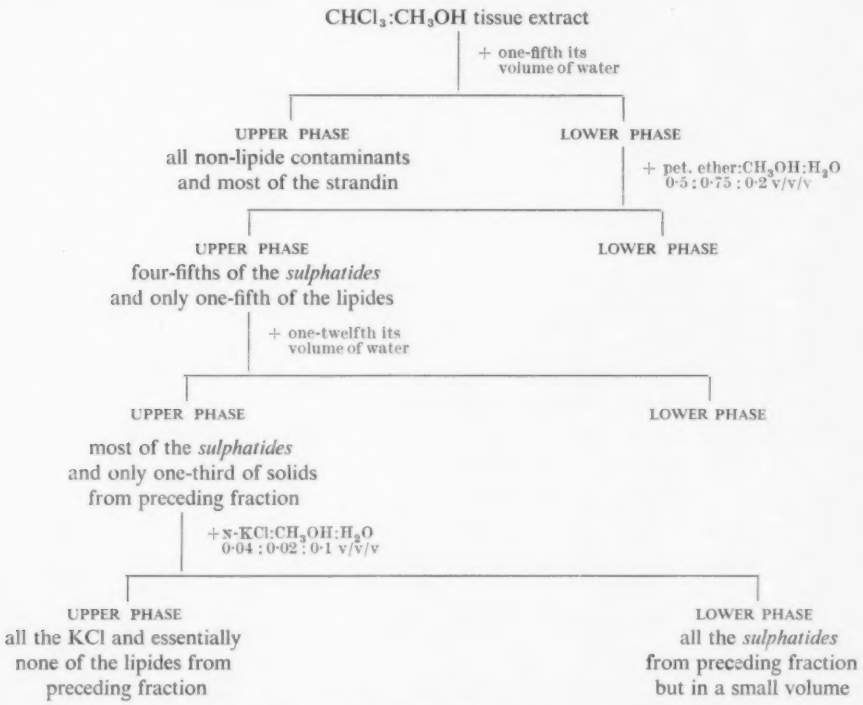
$CHCl_3$:CH_3OH tissue extract
+ one-fifth its volume of water
UPPER PHASE
all non-lipide contaminants and most of the strandin
LOWER PHASE
+ pet. ether:CH_3OH:H_2O 0·5 : 0·75 : 0·2 v/v/v
UPPER PHASE
four-fifths of the *sulphatides* and only one-fifth of the lipides
LOWER PHASE
+ one-twelfth its volume of water
UPPER PHASE
most of the *sulphatides* and only one-third of solids from preceding fraction
LOWER PHASE
+N-KCl:CH_3OH:H_2O 0·04 : 0·02 : 0·1 v/v/v
UPPER PHASE
all the KCl and essentially none of the lipides from preceding fraction
LOWER PHASE
all the *sulphatides* from preceding fraction but in a small volume

FIG. 2.—Method of 'linked distributions' for preparation of sulphatides.

SLOANE STANLEY (1957*a*). Bovine brain white matter is homogenized with a mixture of $CHCl_3$: CH_3OH, 2 : 1 (v/v) in the proportion of 1 g of tissue plus 19 vols. of solvent. The suspension is filtered and to the filtrate is added one-fifth its volume of water. After mixing and centrifugation, two clear phases are obtained. The upper phase, which consists mainly of methanol and water, contains all the non-lipide contaminants and most of the strandin from the original extract. The lower phase, which consists mainly of chloroform, contains all the lipides including the proteolipides, i.e. the sulphatides are quantitatively in the lower phase. The upper phase is removed with suction and the lower phase, which amounts to 60 per cent of the total volume of the system, is collected.

Step 2. To the lower phase thus obtained are added, in succession, methanol, petroleum ether and water in the proportions 1 : 0·75 : 0·5 : 0·2 (v/v). After each addition, the extract is mixed thoroughly; within several minutes after the final mixing, two clear phases have separated. The upper phase contains only one-fifth of the total lipides and, since the sulphatides have a distribution coefficient of 4 : 1 in favour of the upper phase, a marked concentration of sulphatides has occurred in one step. The upper phase also contains a large amount of phosphatidyl serine, since this compound has a distribution coefficient of approximately 3 : 1 in favour of the upper phase. Essentially all of the proteolipides and most of the acetalphosphatides remain in the lower phase. The lower phase, which has a volume approximately 1·2 times that of the upper phase, is removed completely and discarded; the upper phase is collected and its volume noted.

Step 3. To the upper phase thus obtained is added one-twelfth its volume of water. After thorough shaking, the mixture is allowed to stand for several hours or centrifuged briefly. Two clear phases separate. Most of the sulphatides are present in the upper phase along with only one-third of the solids from the previous step. Essentially all the acetalphosphatides carried over from the previous distribution are now present in the lower phase. The lower phase, which has a volume of about one-fourth that of the upper phase, is removed quantitatively and discarded. The upper phase is collected and its volume noted; it is then transferred to a separatory funnel.

Step 4. The upper phase from the previous distribution is mixed with aqueous N-KCl, methanol, and water in the proportions 1 : 0·04 : 0·02 : 0·1 (v/v). After thorough mixing, the system is allowed to stand for several hours, or, if more convenient, overnight. The small amount of lower phase which forms is collected in a glass-stoppered cylinder. It contains most of the solids from the previous upper phase, but in a relatively small volume, amounting to only one-twentieth of the starting volume. Its solids are composed essentially of a mixture of sulphatides and phosphatides; cholesterol, proteolipides and strandin are either absent or present in small amounts (Table 1).

TABLE 1.—COMPOSITION OF SOLUTES OBTAINED FROM THE LOWER PHASE OF STEP 4 IN THE PREPARATION OF SULPHATIDES BY THE METHOD OF 'LINKED DISTRIBUTIONS'

(Data expressed as % of total solutes, unless otherwise noted)

Preparation	I	II	III	IV
Yield (mg/g fresh tissue)	16·2	17·0	17·5	17·45
Sulphur	1·3	1·3	1·2	1·3
Phosphorus	2·30	2·33	2·34	2·33
Sphingomyelin P*	0·24	0·27	0·31	0·26
α-NH_2—N	0·69	—	0·69	0·71
α-NH_2—N (after acid hydrolysis)	0·71	0·70	0·71	0·74
Galactose	8·1	8·4	8·3	8·7
Cholesterol	2·2	2·1	2·3	2·2

* Alkali-stable lipide P (SCHMIDT *et al.*, 1946).

Precipitation of sulphatide

The small volume of lower phase is mixed with petroleum ether and water in the proportions 1 : 0·12 : 0·05 (v/v) and enough methanol is added to make a single phase. The mixture is placed at −10° and, if necessary, more cold methanol is added to maintain a single phase at the low temperature. The precipitate which forms is collected the following day by filtration through a sintered glass funnel of medium porosity and dried in a desiccator over $CaCl_2$. A white, birefringent powder is obtained in the yield of 4 mg/g of fresh white matter. This precipitate accounts for half of the sulphur from the previous fraction; the remainder of the sulphur and essentially all of the phosphorus are present in the filtrate.

Purification of the precipitate

The precipitate, which contains 3 per cent sulphur and 0·2–0·3 per cent phosphorus is referred to below as sulphatide *A* and probably corresponds to BLIX's cerebron sulphuric acid. The last traces of phosphorus can be removed from the compound by three recrystallizations from $CHCl_3 : CH_3OH : H_2O$. The precipitate is dissolved in 100–200 vols. of $CHCl_3 : CH_3OH : H_2O$ 1 : 1·5 : 0·2 (v/v) and the precipitate which forms at −10° is collected and dried. Alternatively, the phosphorus may be removed by adsorption on a Florisil column as described below.

Purification of the filtrate

Numerous attempts to separate the sulphur- and phosphorus-containing compounds in this fraction from one another by solvent fractionation had been unsuccessful. However, by the use of an adsorption column, two clear-cut fractions have been obtained: one is phosphorus-free and contains all the sulphur of the starting material, while the other is a mixture of phosphatides. The procedure is based on the method of RADIN, LAVIN and BROWN (1955), and RADIN, BROWN and LAVIN (1956) for the preparation and determination of cerebrosides. It consists of placing the solids from the filtrate on a Florisil column, eluting the sulphatide with dry $CHCl_3 : CH_3OH$ and then eluting the phosphatide with $CHCl_3 : CH_3OH$ saturated with water. In a typical small-scale preparation, a column of 0·9 cm inner diameter and 40 cm height is used fitted with a ground glass joint at the bottom into which fits a sintered glass plate. Dry Florisil (10 g) is poured into the column to give a column height of 31 cm. The column is washed with about 50 ml $CHCl_3 : CH_3OH$ mixture and the effluent is discarded. Before being placed on the column, the solvents are removed from the filtrate by vacuum distillation at 60°; approximately 500 mg of the solids thus obtained are redissolved in 5 ml 2 : 1 $CHCl_3 : CH_3OH$ mixture and pipetted on to the column. The flask containing the sample is rinsed with two 5 ml portions of solvent, 85 ml more solvent mixture are then permitted to flow through the column at a rate of approximately 1·5 ml/min, and collection of 30 ml aliquots of the effluent is begun as soon as the sample is placed on the column. The successive effluent fractions contain 112·8 mg, 34·2 mg, 1·8 mg and 0·6 mg of solids. The first two fractions contain 0·05 per cent and 0·04 per cent phosphorus respectively and are therefore combined. The column is permitted to dry for several hours after which it is eluted with 100 ml of 2 : 1 $CHCl_3 : CH_3OH$ mixture saturated with water. The eluted fraction contains 347 mg solids and has a phosphorus content of 3·5 per cent.

RESULTS

Analysis of sulphatide A

The results of preliminary studies on the structure of sulphatide *A* are compatible with the assumption that the isolated material is potassium cerebron sulphuric acid (Fig. 1). The analytical data obtained are in agreement with such a hypothesis.

	Theory for K cerebron sulphuric acid (%)	Found (%)	
C*	60·8	60·43 60·34	61·40 61·26
H*	9·93	10·13 10·05	10·10 10·22
N	1·48	1·5	
NH_2—N	0·0	0·08	
S	3·38	3·3	
K	4·12	3·96	
P	0·0	0·03	
Galactose	19·0	18·0	
Reducing sugar	0·0	0·3	

* These analyses were carried out by the Elek Microanalytical Laboratories, Los Angeles, California.

It can be seen that the intact compound contains no free amino nitrogen group, i.e. it gives no reaction with nitrous acid, and that it contains no free reducing group, i.e. it gives no reaction in the Miller–Van Slyke procedure. It must therefore be assumed that the amino group of the sphingosine or sphingosine-like moiety and the aldehyde group of the sugar are each combined. That the sulphur is actually part of the sulphatide molecule and is not present as an inorganic salt which has been carried along during the isolation procedure has been shown by partitioning the sulphatide between $CHCl_3$ and water; the aqueous phase contains about 0·1 per cent sulphur.

The liberation of these various groups by 6 N-HCl at 100° has been studied as follows: Samples of sulphatide *A* were heated for varying lengths of time with 6 N-HCl at 100°. After the hydrolysates had been cooled and neutralized, estimations of reducing sugar and amino nitrogen were carried out on the entire hydrolysate. Estimations of galactose and sulphur were carried out on the aqueous phase obtained after partition of the hydrolysate between $CHCl_3$ and 6 N-HCl.

It has been found that all the nitrogen reacts as amino nitrogen* after treatment with 6 N-HCl for 30 min. Essentially all the galactose is liberated in water-soluble form in 6 min with the appearance of reducing action paralleling the release of galactose. It thus appears that the glycosidic linkage is more labile to treatment with acid than is the combination of the amino group with the fatty acid. Further study of these hydrolysates by paper chromatographic techniques, which were kindly carried out by Dr. S. BOGOCH, showed a single spot which corresponded to the position of galactose; glucose appeared to be absent. A negative Elson–Morgan reaction (MORGAN and ELSON, 1934) indicated the absence of amino sugars. Hence, galactose appears to be the only sugar present.

* Since it is known that sphingosine reacts slowly with nitrous acid (LEVENE and JACOBS, 1912), the reaction times used for these analyses were 3 times those used normally.

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Attempts to prepare products of partial hydrolysis of sulphatides

Ceramide and psychosine are the products of partial hydrolysis of cerebrosides (DEUEL, 1951). Ceramide is the term applied to a fatty acid–N–sphingosine compound; it is free of galactose and has the carboxyl group of the fatty acid and the amino group of the sphingosine combined with one another in an N-acyl linkage. Psychosine is constituted by galactose and sphingosine combined in glycosidic linkage and, accordingly, contains no free reducing group; however, all its nitrogen occurs as free amino nitrogen and should react with nitrous acid in the VAN SLYKE procedure (1929). Since it appeared that sulphatide *A* was a sulphuric acid ester of a cerebroside, it seemed reasonable to expect that ceramide and psychosine could be obtained by the procedures used by KLENK to prepare these substances from phrenosin (1926). For the preparation of ceramide, the sulphatide was treated with a mixture

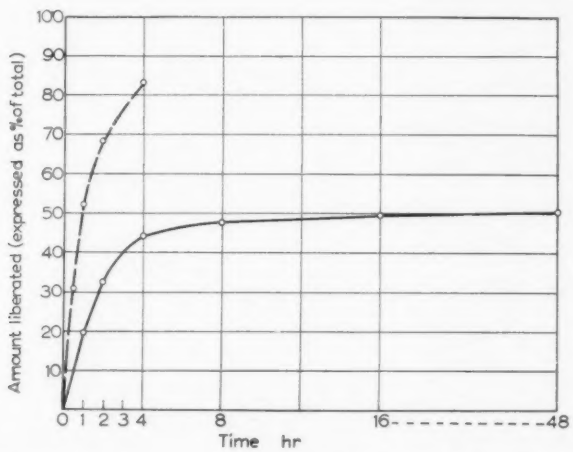

FIG. 3. Release of inorganic sulphur from sulphatide *A* in the presence of glacial acetic acid at 100°.
——— Release of NH_2—N as estimated by the VAN SLYKE nitrous acid method (1929).
– – – – – Liberation of galactose. Since free galactose is destroyed by alkali, these figures were calculated from the amount of galactose which remained unhydrolysed.

of glacial acetic acid : 10% H_2SO_4 (10 : 1 v/v) in a boiling-water bath for 42 min; no ceramide was found in the hydrolysate. For the preparation of psychosine, sulphatide *A* was hydrolysed with saturated $Ba(OH)_2$ for 7 hr in a boiling-water bath and attempts were made to crystallize psychosine from the hydrolysate; little or no psychosine was obtained. Further study of the effects of hydrolysis revealed that after the above-mentioned acid hydrolysis, the sulphatide had been completely split into its component parts. On the other hand, hydrolysis with saturated $Ba(OH)_2$ resulted in a relatively slow appearance of free amino nitrogen with essentially no further increase in free amino nitrogen after hydrolysis for 4 hr; even after 48 hr, about half of the nitrogen was still combined in a form which did not react directly with nitrous acid (Fig. 3). Furthermore, contrary to KLENK's observations on cerebrosides, the galactosidic linkage of the sulphatide was cleaved relatively easily by the alkali with a consequent destruction of more than 80 per cent of the galactose in 4 hr.

Hydrolysis of sulphatide by glacial acetic acid

In the course of the studies on the isolation of the products of partial hydrolysis of sulphatides, it became apparent that glacial acetic acid alone had an effect on sulphatide *A*. In order to obtain information on this point sulphatide *A* was treated with glacial acetic acid at 100° for varying lengths of time and the release of the various components followed. It was found that (a) the sulphur is combined in the sulphatide molecule; hydrolysis for 90 min is required to release the maximum amount of the sulphur as inorganic sulphate (Fig. 4); and (b) after the removal of essentially all of the sulphur, the cerebroside portion of the molecule appears to remain intact.

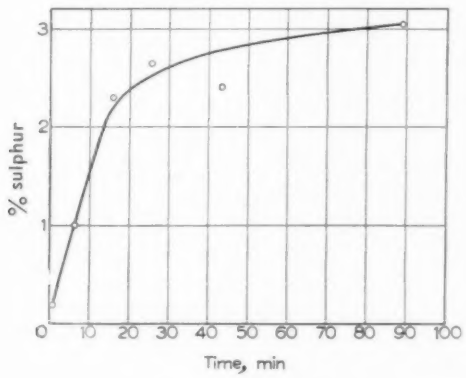

FIG. 4. Hydrolysis of Sulphatide A by saturated $Ba(OH)_2$ at 100°.

A typical experiment is carried out as follows: To 10 mg of sulphatide *A* in a test-tube is added 0·3 ml glacial acetic acid and the tube is placed in a vigorously boiling water bath. At the end of the required time, the tube is removed, cooled under running tap water and 2·7 ml water and 3 ml $CHCl_3$ are added. The contents of the tube are mixed thoroughly with a stirring rod for 1 min, the rod is rinsed with two or three drops of water, and the tube centrifuged for 20 min. As much of the resultant upper phase as possible is removed with a pipette and transferred to an evaporating dish. Water (3 ml) is added to the tube, the contents of the tube mixed as described above and centrifuged for 30 min, or until two clear-cut phases separate. The upper phase is removed and combined with the first aqueous phase. The washing procedure is repeated and the combined aqueous phases are dried. The residue is redissolved in water and transferred to a volumetric flask. After diluting to the mark, aliquots are taken both for precipitation of sulphuric acid with benzidine reagent and for estimation of galactose. After the addition of enough methanol to make a single phase, the lower phase can be analysed for cerebrosides by appropriate methods.

When the above procedure was applied to the fraction containing both sulphur and phosphorus it was found that, under conditions where essentially all the sulphur of sulphatide *A* was released as inorganic sulphate, no sulphur was released from the fraction containing both sulphur and phosphorus. Although at first this observation was interpreted as evidence for the existence of two different sulphatides, it was later found that the failure of acetic acid to liberate sulphuric acid from the fraction containing both sulphur and phosphorus was due to the presence of phosphatide(s). The following observations have been made: (a) After passage of the fraction

containing both sulphur and phosphorus through a Florisil column, the phosphorus-free sulphatide thus obtained is hydrolysed by acetic acid as is sulphatide *A*. (b) The addition to sulphatide *A* of either the fraction containing both sulphur and phosphorus, or the phosphatide mixture eluted from a Florisil column, decreases or completely suppresses the release of sulphuric acid in the presence of acetic acid, the magnitude of the effect being related to the amount of phosphatide(s) added. (c) The effect of the presence of phosphatide(s) appears to be overcome by the use of larger amounts of acetic acid in the hydrolysis procedure.

Analysis of fractions obtained after Florisil treatment

The fraction eluted from the Florisil column with dry $CHCl_3$:CH_3OH contains all the sulphur present in the material placed on the column and is obtained in a yield of 4·2 mg/g fresh white matter. The analysis of the material is as follows: sulphur, 2·1%; phosphorus, 0·0–0·05%; nitrogen, 1·44%; galactose, 16·0%; cholesterol, 10·6%; strandin, 1·2%; moles galactose : atoms sulphur, 1·4 : 1. The fraction thus appears to be a sulphatide plus non-phosphatide components (cerebrosides and cholesterol) which may be present as contaminants or as part of the sulphatide molecule. Thus far, there is no evidence that this sulphatide is different from sulphatide *A*.

The fraction eluted from the column with $CHCl_3$:CH_3OH saturated with water is a mixture of phosphatides. It is a slightly waxy, light tan material obtained in a yield of 9·8 mg/g fresh white matter. The analysis of the material is as follows: phosphorus, 3·57%; sulphur $<0{\cdot}1$%; galactose, 0·86%; nitrogen, 1·51%; α-amino acid nitrogen 0·86%; cholesterol, 0; acetalphosphatides, 3%. Over half of the phosphatide mixture comprises phosphatidyl serine; the bulk of the remainder is still to be identified.

DISCUSSION

The principle of 'linked distributions' has widespread potential application to lipide fractionation procedures since it is based on a physical property of the individual lipide being studied, i.e. its distribution in a biphasic system. The distribution coefficient of a lipide is independent of the lipide composition of the mixture at the dilutions indicated and therefore the procedure is completely reproducible, even with different starting materials or with samples of various sizes. The use of a related series of biphasic systems has the further advantage that at no time is it necessary to concentrate or evaporate to dryness any of the phases. The particular procedure of 'linked distributions' described in this paper is an empirical method which was the result of work aimed at finding optimum conditions for the quantitative isolation of sulphatides by a simple and mild procedure. The development of each step involved a number of pilot fractionations carried out to devise a biphasic system, using only neutral solvents, in which tenacious lipide emulsions did not form, and in which the sulphatides had a distribution coefficient markedly in favour of one of the phases. The yields reported in this paper can be increased by appropriate reprocessing of discarded fractions, and the bulk of the lipide sulphur can be accounted for. In the final step in the series of 'linked distributions', the sulphatides are obtained in a small volume amounting to only one-twentieth that of the starting volume. This is made possible by taking advantage of a previous observation that the distribution of certain lipides, i.e. acidic lipides, between two phases of a solvent system is affected by the presence or absence of

inorganic cations (FOLCH, LEES and SLOANE STANLEY, 1957). Upon the addition of inorganic salts, all the acidic lipides are present in the lower, highly non-polar phase. By devising a two-phase system in which the lower phase constitutes only a small part of the total volume, it is possible to reduce markedly the volume of solvents being handled, with little or no loss of sulphatides. The complete reproducibility of the procedure of 'linked distributions' is shown in Table 1 which gives the analysis of the final fraction obtained in the course of four different sulphatide preparations. When this fraction was placed at $-10°$, a voluminous sulphur-rich precipitate separated, but, because of its gelatinous nature, collection by filtration was practically impossible. However, the relatively slight changes in solvent composition described in the procedure ('precipitation of sulphatide') produce a more granular precipitate which is mechanically manageable.

SUMMARY

A method is described for the isolation of sulphatides from bovine brain white matter in good yield by a simple, mild and reproducible procedure. It is based on the distribution of lipides between the two phases of a series of related solvent systems. Two main fractions are obtained. One fraction is an essentially pure sulphatide (sulphatide *A*); the other contains both sulphur and phosphorus and can be separated into a phosphorus-free sulphatide fraction and a phosphatide mixture by passage through a Florisil column. Some observations on the structure of sulphatide *A* are discussed.

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THE BLOCKING EFFECT OF γ-AMINOBUTYRIC ACID (GABA) AND THE ACTION OF RELATED COMPOUNDS ON SINGLE NERVE CELLS*

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IN 1953 FLOREY extracted from mammalian nervous tissue an inhibitory factor which had a blocking action on the stretch receptors of crustacea described by ALEXANDROWICZ (1951). Lately, the composition of the inhibitory factor was analysed and its activity attributed to its content of γ-aminobutyric acid (GABA) (BAZEMORE, ELLIOTT and FLOREY, 1957). In somewhat similar studies, HAYASHI (1956) and HAYASHI and NAGAI (1956), investigated various compounds, mostly amino acids, which exerted excitatory and blocking effects on the cortex and on isolated muscle. The most effective blocking compound was found to be γ-amino-β-oxybutyric acid. Since then GABA and related compounds have been tested for their action on cortical neurones (PURPURA, GIRADO and GRUNDFEST, 1957, 1958; IWAMA and JASPER, 1957) and the mechanisms of GABA action on crustacean stretch receptors have been studied by KUFFLER and EDWARDS (1958).

Before the physiological interest in GABA arose, this compound had been known biochemically. It is present in the central nervous system in relatively large amounts (AWAPARA, LANDUA, FUERST and SEALE, 1950; ROBERTS and FRANKEL, 1950; UDENFRIEND, 1950) and there are indications that it may play a role in cerebral metabolism, especially in connexion with glutamic acid turnover (cf. ROBERTS, 1956, for a review). It has also been found chromatographically in crustacean nerves (BURGEN and KUFFLER, unpublished).

The present report is a continuation of studies dealing with inhibitory synaptic mechanisms in isolated nerve cells of stretch receptors in the crayfish and lobster. GABA action has been found to be similar in many important aspects to that of the normally occurring inhibitory neural transmitter. It is thought that GABA, as well as the transmitter, blocks by selectively increasing the movement of specific ions across the nerve cell membrane. This increased ionic conductance interferes with the processes which lead to initiation of conducted nerve impulses (KUFFLER and EDWARDS 1958). It has become of interest to determine some of the factors which make the GABA molecule physiologically active. A study was therefore made of some related compounds in which the end-groups, the chain length and the side groupings were changed. The NH_2 and COOH end-groups were found essential for the blocking action, which was optimal when the end-groups were separated by three carbon atoms. Lengthening or shortening the chain length decreased effectiveness, as did addition of side chains. The action of some of the compounds used has been previously studied on

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the crayfish stretch receptor (ELLIOTT and FLOREY, 1956; BAZEMORE, ELLIOTT and FLOREY, 1957).

It has been found (KUFFLER and EDWARDS, 1958) that GABA is inactivated by the tissue. This inactivation process apparently differs significantly among closely related compounds and these differences are analysed.

METHODS

The technique has already been described in detail by KUFFLER and EDWARDS (1958). Slowly adapting stretch receptors from the thorax or from one of the abdominal segments of crayfish (*Orconectes virilis, Procambarus alleni*) were placed in a fine groove, ending in two circular reservoirs, ground into a glass slide. Some compounds were also tested on the lobster receptor (*Homarus americanus*) which proved a less sensitive preparation. All specific findings in this paper refer to crayfish. The crayfish sensory cell was normally surrounded by VAN HARREVELD's solution (1936) to which 10 moles/l. sodium maleate buffer, pH 7·2, was added. The cell's discharges, resting potentials and, when required, its inhibitory synaptic potentials were recorded. Solutions were readily replaced without appreciably changing recording conditions. The total capacity of the test chamber was about 0·05 ml. In many experiments only the discharge frequency of the receptor was measured by an electronic counter. In others the membrane potentials were also followed by measuring the potential differences between the axon hillock region and a point on the axon several millimetres more central. To make certain that the blocking drug actions took place in the dendrites or cell body, the agents were applied to the axons where conduction was found unimpaired. Acceleration of discharge in nerve cells by test solutions was not studied in detail because it was difficult to determine whether excitatory substances acted directly on the nerve cell or indirectly by causing contraction in the receptor muscle.

Numerous compounds not reported here were tested but are omitted because their effects do not seem relevant to the present problem. The number of trials for each substance varied. Usually the threshold concentration for blocking a sensory discharge of 5–8 per sec for 20–30 sec was first determined and this test was then repeated several times. The blocking potency of each compound was related to that of GABA, tested on the same cell, and is expressed as the ratio of the concentration of the test solution to that of GABA. If the first results on a cell were consistent with studies on two subsequent preparations, further tests were not made unless other special problems were studied, for instance effects on the neural inhibitory process or inactivation. Each figure given in the tables represents one or more trials on a single preparation. The threshold blocking concentration for GABA was usually 2×10^{-5} moles/l. but fluctuations over the range of 5×10^{-6} and 3×10^{-5} moles/l. were found in different cells. In the course of several hours various drugs were checked several times on one cell since most effects were readily reversible within a few minutes. Problems of diffusion within the tissue are greatly reduced in the isolated cell preparations.

RESULTS

Effect of chain length of ω-amino acids. A series of ω-amino acids were tested for blocking action of sensory discharge. Table 1 shows the effectiveness of these compounds in terms of the threshold concentration of the substance relative to the threshold concentration of GABA. The C_3 and C_5 compounds were about equally less potent than GABA as previously found by BAZEMORE, ELLIOTT and FLOREY (1957). The activity of the C_2 and C_6 derivatives was reduced still more. The loss in potency due to addition or subtraction of one carbon atom is quite noticeable. It therefore seems that for optimal blocking effect the end groups should be three carbons apart.

Guanido acids. Guanidoacetic acid was found to be only a little less effective than GABA (Table 2). It resembles GABA in several ways. The distance between the terminal amine group and the carboxyl group is almost the same, and both compounds have a positive group at one end and a negative group at the other end (the ionized carboxyl group). However, the terminal amino group of guanidoacetic acid is less positive than that of GABA because of the resonance within the guanido group.

TABLE 1

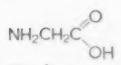
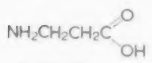
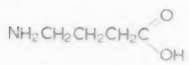
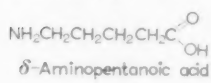
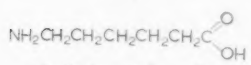
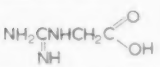
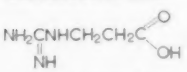
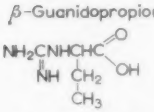
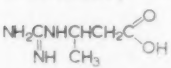
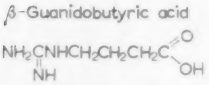
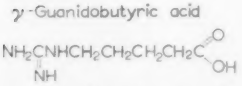
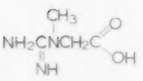
Amino acid	Ratio of blocking concentration of amino acid to blocking concentration of GABA
 Glycine	1300, 1300
 β-Alanine	20, 20, 22
 γ-Aminobutyric acid	1
 δ-Aminopentanoic acid	12, 13, 13, 13, 17
 ε-Aminohexanoic acid	75, 90, 150

TABLE 2

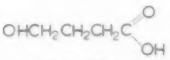
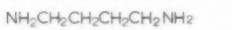
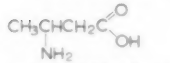
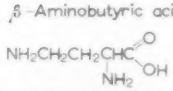
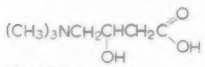
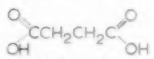
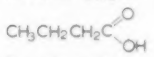
Guanido acid	Ratio of blocking concentration of guanido acid to blocking concentration of GABA
 Guanidoacetic acid	1·2, 1·4, 1·4, 3·0
 β-Guanidopropionic acid	1·2, 1·2, 1·2, 1·4, 1·6, 1·9
 α-Guanidobutyric acid	260, 260, 260, 330
 β-Guanidobutyric acid	7, 7, 7, 12, 13
 γ-Guanidobutyric acid	3, 5, 7, 8, 9, 10, 10, 13
 δ-Guanidopentanoic acid	190, 190
 Creatine	750, 1500

Another difference in the properties of the two end groups will be pointed out below.

The next longer analogue, β-guanidopropionic acid, was as effective as guanidoacetic acid. The reason for this is not obvious in the light of a theory based on chain length alone.

With further increase in chain length there was a loss of effectiveness. The fact that γ-guanidobutyric acid is more effective than ε-aminohexanoic acid indicates that

TABLE 3

Compound	Ratio of blocking concentration of compound to blocking concentration of GABA
 γ-Hydroxybutyric acid	25, 50, 100, 100
 1:4-Diaminobutane	900, 900, 1200
 β-Aminobutyric acid	250, 500, 1000
 2:4-Diaminobutyric acid	200, 200, 250
 Carnitine	Accelerated
 Succinic acid	Accelerated
 Butyric acid	Accelerated

its activity may be attributable to the positivity of the N attached to the γ carbon; if its activity were due to the terminal amine group alone, the effectiveness of these compounds should be similar.

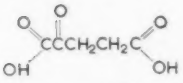
It is of interest that γ-guanidobutyric acid has been found in the mammalian brain and other tissues (IRREVERRE, EVANS, HAYDEN and SILBER, 1957). The synthesis of this substance has been demonstrated in mammals by PISANO, MITOMA and UDENFRIEND (1957) and in invertebrates by THOAI, RIOCHE and ROBIN (1953).

Effect of neutral side chains. The effect of addition of neutral side chain groups on the effectiveness of a compound is illustrated in Tables 1, 2 and 3. β-Guanidobutyric acid (or β-methyl-β-guanidopropionic acid) was less effective than β-guanidopropionic acid. Similarly β-aminobutyric acid (β-methyl β-alanine) was less potent than β-alanine. α-Guanidobutyric acid (α-ethyl guanidoacetic acid) and creatine (N-methyl-guanido acetic acid) were both less potent than guanidoacetic acid. Thus addition of neutral groups on the carbon skeleton reduced the effectiveness of the compounds.

Effect of different four-carbon compounds. The alteration of the end groups of GABA was investigated in the series shown in Table 3. Substitution of a hydroxyl group for the amino group in GABA reduced blocking activity by about 50 times. The groups sterically are similar, but under the present experimental conditions the

amino group carries a positive charge and the hydroxyl group is neutral. The lactone of γ-hydroxybutyric acid was without effect at 10^{-2} moles/l. The lack of inhibitory action of butyric and succinic acids is further evidence for the necessity of a positive group at one end.

TABLE 4. –BLOCKING ACTION OF MISCELLANEOUS COMPOUNDS

Compound	Ratio of blocking concentration of compound to blocking concentration of GABA
$NH_2CH_2CH_2SO_3H$ Taurine	140, 160, 320
$NH_2CH_2CH_2CH_2CH_2NHCNH_2$ (NH) Agmatine	150, 375, 600, 750
 α-Ketoglutaric acid	Accelerated

The importance of the carboxyl group was shown by the low potency of 1:4-diaminobutane (see also agmatine, Table 4). The addition of an amine group to the α carbon of GABA decreased the activity. Further, 6×10^{-4} moles/l. carnitine (γ-trimethylamino-β-hydroxybutyric acid) had no blocking action, while 6×10^{-3} moles/l. accelerated it (Table 3). γ-Amino-β-hydroxybutyric acid, which was found very effective in HAYASHI's (1956) studies was not tested; however, BAZEMORE, ELLIOT and FLOREY (1957) report it to be about one-half as potent as GABA on the stretch receptor.

Other compounds. Taurine (2×10^{-3} moles/l.) had a blocking action and is seen to have a charge distribution similar to β-alanine (Table 4). It was less effective than β-alanine, possibly because of the greater size of the SO_3^- group compared to the COO^- group. Agmatine (1-amino-4-guanidobutane) was interesting in that it exhibited some blocking activity although it lacks a negatively charged group. In the presence of 7×10^{-5} moles/l. α-ketoglutaric acid the frequency of discharge was increased.

Inactivation and Desensitization

In the presence of an appropriate level of GABA, the block of the cell is followed by recovery. The adaptation reported by FLOREY (1957) after factor *I* application seems to reflect the same phenomenon. In Fig. 1(B) the cell discharge of 8 per sec was blocked completely for 60 sec; 90 sec after the cell first started firing again, the discharge rate was up to 3 per sec. If the solution in the groove was then stirred the cell was again blocked for about 60 sec and then recovered with a time course similar to that following the initial application. This mixing effect could be repeated as often as desired on a given cell, and was a property of all of the more than fifty cells used in these tests. If the stirring was continued the block was maintained. In a previous paper, in which this finding was first reported (KUFFLER and EDWARDS, 1958), it was concluded that GABA was inactivated by the tissue and over a certain concentration range the rate of inactivation was greater than the rate at which fresh GABA diffused in from the bulk phase. The term 'inactivation' indicates not only a process in which GABA might be chemically altered so as to become ineffective, but also included any

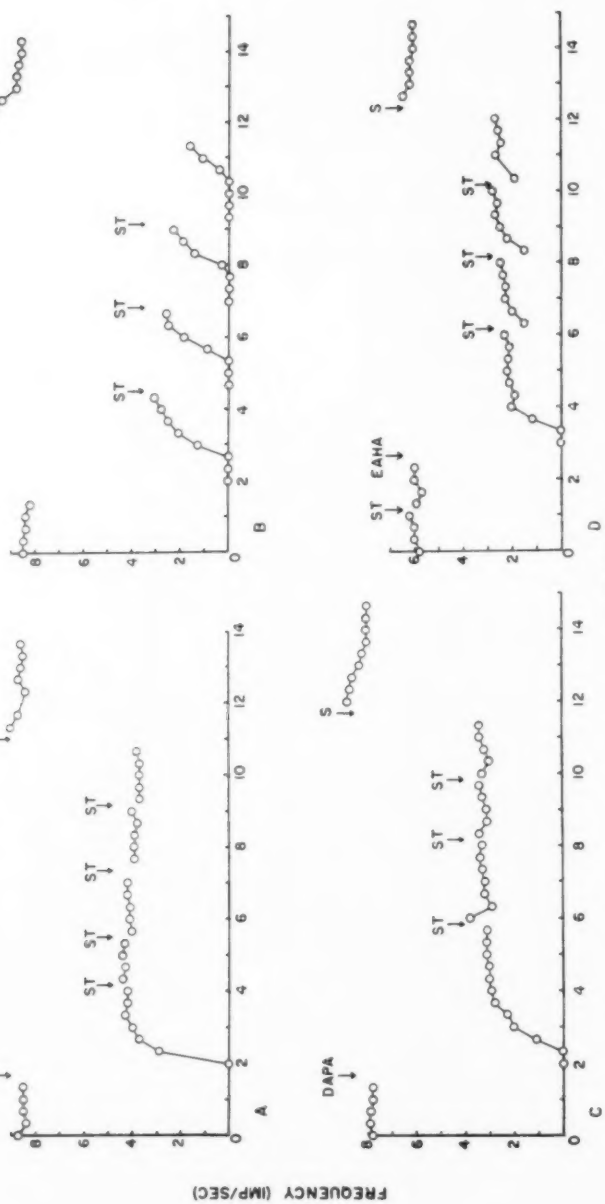


FIG. 1.—Effect of various ω-amino acids on the discharge frequency of a stretch receptor nerve cell. Solutions added at first arrow, solutions stirred when indicated (ST), and fresh bathing solution added at (S). (A): $4{\cdot}4 \times 10^{-4}$ moles/l. β-alanine (three carbons). (B): 2×10^{-5} moles/l. γ-aminobutyric acid (four carbons). (C): $2{\cdot}6 \times 10^{-4}$ moles/l. δ-aminopentanoic acid (five carbons). (D): 3×10^{-3} moles/l. ε-aminohexanoic acid (six carbons). All solutions blocked when first applied. Stirring did not block in (A), (C) and (D). Block re-established by stirring in (B).

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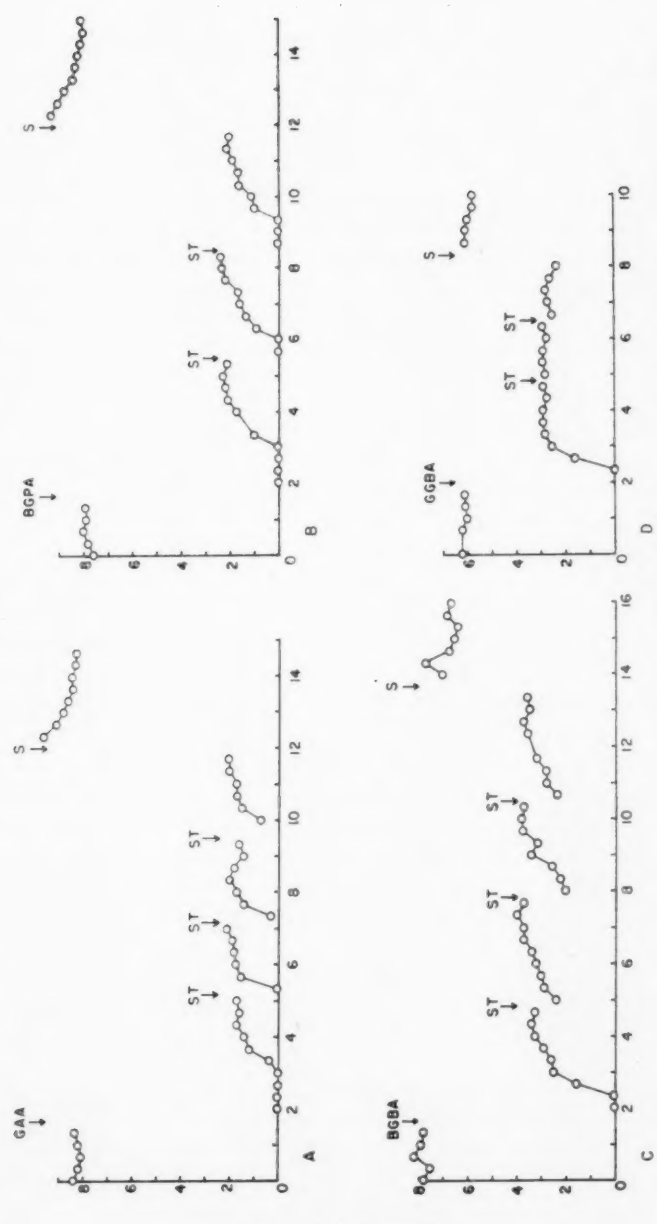

FIG. 2.—Effect of various guanido acids on the discharge frequency of a stretch receptor nerve cell. Solutions added at first arrow stirred when indicated (ST), and fresh bathing solution added at (S). (A): $2{\cdot}5 \times 10^{-5}$ moles/l. guanidoacetic acid. (B): $2{\cdot}3 \times 10^{-5}$ moles/l. β-guanidopropionic acid. (C): $1{\cdot}4 \times 10^{-4}$ moles/l. β-guanidobutyric acid. (D): $2{\cdot}5 \times 10^{-4}$ moles/l.γ-guanidobutyric acid. All solutions blocked when first applied. Stirring ineffective in blocking in (D), progressively more effective in blocking in (C), (A) and (B).

process by which GABA might be removed from its site of action by the tissue. ELLIOTT and VAN GELDER (1958) have in fact shown an uptake of GABA by brain slices. Uptake by the tissue and chemical alteration would have the same result, i.e. reduction in the quantity of GABA in the immediate neighbourhood of the receptor, and therefore attenuation of the block due to GABA. Stirring or replacing the solution restored the GABA level in the vicinity of the receptor to that of the bulk phase, so the discharge was blocked to the same extent as during the original application of the drug. Inactivation of GABA and of several guanido compounds is shown in Figs. 1 and 2.

There was no inactivation, or the effect was much less pronounced than in the case of GABA, when other amino acids were applied to the cells. Although the receptors were blocked with suitable concentrations and recovery occurred afterward, stirring did not change the firing rate, i.e. mixing the solution did not re-establish the block of the cell. Furthermore, replacing the bathing fluid with fresh solution, containing the same concentration of the agent which at first blocked the discharge, was not effective (not illustrated). Since it appears that the sensitivity of the cell to these agents decreased in the presence of drugs, the term desensitization will be used. A rinse in normal bathing solution restored the original discharge rate and blocking effects could be obtained once more. Although similar observations are common, little is known of the mechanisms involved in the loss of sensitivity. A clear recent demonstration of desensitization is the finding that the depolarization of the membrane at the neuromuscular junction set up by acetylcholine diminishes during continued acetylcholine application (KATZ and THESLEFF, 1957). Desensitization has also recently been reported by KUFFLER and EDWARDS (1958) with γ-guanidobutyric acid.

Effect of ω-amino acids. The typical inactivation as seen in Fig. 1(B) during GABA action has already been mentioned. The complete re-establishment of the block by stirring was also quite typical. This indicates that no appreciable desensitization takes place during recovery after GABA application, for otherwise successive stirrings should become much less effective. Washing in fresh bathing solution restored the original discharge rate after an initial 'rebound' to 10 per sec. In Fig. 1(A) the same procedure was followed with the three carbon compound, β-alanine. The initial block of discharge produced by $4{\cdot}4 \times 10^{-4}$ moles/l. was followed by a recovery to half the original discharge rate within $1\frac{1}{2}$ min. Four separate stirrings of the solution did not appreciably change the discharge frequency which remained near 4 per sec until the solution was replaced by fresh solution. δ-Aminopentanoic acid (five carbons) acted in a similar way (Fig. 1C) at a similar concentration of $2{\cdot}6 \times 10^{-4}$ moles/l. ε-Aminohexanoic acid (six carbons) in a much greater concentration of $3{\cdot}3 \times 10^{-3}$ moles/l. (Fig. 1D) did show a stirring effect which, however, was not pronounced and of quite short (20 sec) duration. A control stirring in ringer solution is shown in this preparation.

Although the breakdown of GABA in mammalian brain seems to proceed by transamination (cf. ROBERTS, 1956) there is no evidence on this point in crustacea. To test the hypothesis that inactivation proceeds by removal of the amino group, an amine oxidase inhibitor, 10^{-4} moles/l. Marsilid (*iso*propyl *iso*nicotinic hydrazide) was added to a blocking concentration of GABA. No significant change in GABA inactivation was found, suggesting that if alteration of the GABA occurs, the route is not via an amine oxidase. In a similar experiment, a preparation was bathed in another amine oxidase inhibitor, *iso*nicotinic hydrazide ($7{\cdot}5 \times 10^{-3}$ moles/l.). The inhibitory

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nerve was stimulated and the drug did not change the synaptic inhibitory potential. Thus the naturally occurring inhibitory transmitter is not broken down by an amine oxidase, for if it were, the synaptic inhibitory potential would be greatly prolonged. An example of the alteration of transmitter action by block of an enzyme is found at the neuromuscular junction; anticholinesterases prevent the hydrolysis of acetylcholine, thereby prolonging the endplate potential.

Guanido compounds. The effects of stirring on the action of several guanido compounds is shown in Fig. 2. With guanidoacetic acid ($2{\cdot}5 \times 10^{-5}$ moles/l.) there is a definite block with stirring, but the duration is shorter than with the initial application (Fig. 2A). With $2{\cdot}3 \times 10^{-5}$ moles/l. β-guanidopropionic acid, stirring the solution blocked the discharge (Fig. 2B) similarly to the initial application much like GABA in Fig. 1(A). This contrasts with the block set up by $1{\cdot}4 \times 10^{-4}$ moles/l. β-guanidobutyric acid which showed little effect on stirring (Fig. 2C). The same is true of γ-guanidobutyric acid (Fig. 2D) as previously reported (KUFFLER and EDWARDS, 1958).

The stirring effect was tried on various other compounds. Of interest may be the absence of inactivation with γ-hydroxybutyric acid and with agmatine.

Action on membrane potentials and on neural inhibitory processes

Stimulation of the inhibitory axon of a receptor cell gives a small transient change in the membrane potential. In a stretched depolarized cell the inhibitory synaptic potentials hyperpolarize, while in a relaxed receptor the inhibitory process may cause no potential change or a small depolarization. The explanation for this is that the inhibitory process transiently drives the membrane potential towards an equilibrium level (FATT and KATZ, 1953; KUFFLER and EYZAGUIRRE, 1955). It has previously been shown that the block of discharge in a stretched receptor by GABA and γ-guanidobutyric acid is accompanied by a hyperpolarization, while in a relaxed cell these agents may not cause membrane potential changes although the inhibitory process is fully active (KUFFLER and EDWARDS, 1958). In the present tests it was found that various blocking agents acted in a similar manner, causing either a hyperpolarization or no appreciable resting potential change. These agents included: β-alanine, δ-aminopentanoic acid, ε-aminohexanoic acid, guanidoacetic acid, β-guanidopropionic acid, α-guanidobutyric acid and agmatine. It should be realized that block can also be produced by excessive depolarization. Such an action can be recognized because it is generally preceded by an increased discharge frequency.

It was also found that the following compounds reduced the height of the inhibitory synaptic potentials and shortened their duration: β-alanine, δ-aminopentanoic acid, guanidoacetic acid, β-guanidopropionic acid, β-guanidobutyric acid and agmatine.

The hyperpolarization of a depolarized cell by the neural inhibitory transmitter, as well as by the present group of blocking agents, is regarded as due to an increased conductance to specific ions which have their equilibrium level near the resting potential of the cell. The increased conductance during block is responsible for the reduction of the height of the inhibitory synaptic potential and for the shortening of the time and space constants of the membrane. The latter is reflected in the short duration of the inhibitory potential (for details see KUFFLER and EDWARDS, 1958). Although the

inactivation and the densensitization reactions of the different blocking agents were not identical, their membrane action seems to be similar.

It has previously been shown that GABA and γ-guanidobutyric acid do not appreciably affect the axon membrane. The site of block is confined to the dendrites and cell body. Since none of the other amino acids tested interferes with axon conduction and during block repolarizes the cell body and dendrite region, it is concluded that this is the site of block also.

PURPURA *et al.* (1957) reported that in the cat's cortex ε-aminohexanoic acid and ω-amino-octanoic acid selectively block the inhibitory synapses. The shorter ω-amino acids, however, 'inactivate' excitatory synapses. The blocking concentrations for sensory discharges of receptor cells of ε-aminohexanoic acid was near 8×10^{-3} moles/l., while at a concentration of 7×10^{-3} moles/l. ω-amino-octanoic acid was not effective. With concentrations just below block the effectiveness of inhibitory axon stimulation remained practically unimpaired. In the crayfish receptor neurone, therefore, these long-chain amino acids do not exert a selective blocking action on the inhibitory synapses. More recently PURPURA *et al.* (1958) found that, whereas guanidoacetic acid blocks as does GABA, γ-guanidobutyric acid blocks inhibitory synapses, as does ε-aminohexanoic acid. No such difference in action between the shorter and longer homologues of either series was seen in the stretch receptor cells.

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DISCUSSION

The evidence presented here again raises the question of whether GABA is a naturally occurring inhibitory transmitter. It has been shown that it is the most potent blocking agent of all the compounds tested. GABA has been found chromatographically in crustacean nerve but is not confined to inhibitory neurons (BURGEN and KUFFLER, unpublished). There exists a system to inactivate it, either by removal or alteration. Finally its effects on certain membrane phenomena are similar to those of the naturally occurring inhibitory transmitter (KUFFLER and EDWARDS, 1958). All this evidence, however, does not show that GABA normally has an inhibitory role. This can be proved only by showing that GABA, or some related substance, is actually released in amounts adequate to block nerve cells. Contra-indicating GABA's being the inhibitory transmitter is the fact that its action is not confined to subsynaptic areas, since it also blocks the seventh median thoracic receptor of the lobster which apparently does not carry inhibitory synapses (KUFFLER and EDWARDS, 1958). Furthermore, inhibitory substances other than GABA have been found in nerve extracts (BURGEN and KUFFLER, unpublished).

One reason for testing the above compounds was to gain information as to the way in which the blocking substances are inactivated by the receptor. The stirring effect with GABA indicates that this substance is inactivated; however, as noted, inactivation may be either an active uptake or chemical alteration, for by either route the local concentration of GABA would be reduced below that in the bulk phase. ELLIOTT and VAN GELDER (1958) have reported that brain slices take up GABA to the extent of inside–outside concentration ratios of 40 or more. In the present case GABA may also be concentrated by the receptor muscle, a possibility which was not studied. If, however, the inactivation is by chemical alteration, the route is not at all certain, except that inactivation via an amine oxidase system is unlikely.

It has been shown that GABA can be transaminated in the presence of α-ketoglutarate, yielding glutamate and succinic semialdehyde (BESSMAN, ROSSEN and LAYNE, 1953), but it remains open whether this reaction occurs in the inactivation of GABA in intact cells.

The threshold concentration studies seem to be of some interest in the problem of structure–function relationship of inhibitory compounds. They also, however, show that relatively great sensitivity alone does not solve the problem of the identity of transmitters, since GABA, guanido-acetic acid and β-guanidopropionic acid are almost equally effective.

It should be noted that ω-amino acids and guanido acids tested on the stretch receptor all blocked, although at different concentrations. The mechanism of the blocking action seems to be similar and is satisfactorily explained by the increased ionic conductance of the membrane. This uniform action is in contrast to the report of PURPURA *et al.* (1957, 1958). In their tests the shorter ω-amino and ω-guanido acids block excitatory activity while the longer homologues (starting with ε-aminohexanoic and γ-guanidobutyric acid) apparently inactivate inhibitory synapses. The basis of these differences is of interest.

SUMMARY

(1) The blocking action of γ-aminobutyric acid (GABA) and of various other amino acids was analysed on the isolated stretch receptor cells of crayfish.

(2) All the amino acids which block sensory discharges in cells repolarized the depolarized cell membrane towards its resting potential level. It is concluded that the common mechanism of the various amino acids consists in increasing the flow of specific ions across the membrane of dendrites and of the cell body. This action resembles the mechanism by which the neural inhibitory transmitter acts on the sensory cell.

(3) The NH_2 and COOH end groupings were essential for the blocking action which was optimal if the end groups were separated by three carbon atoms. Lengthening or shortening the chain length decreased effectiveness. Addition of neutral side groups also raised the concentration necessary for block.

(4) Some guanido acids may block sensory discharges almost as effectively as GABA.

(5) Processes of inactivation of blocking agents have been analysed. A cell may gradually regain its activity while it is still surrounded by some of the blocking compounds. It is suggested that these amino acids are chemically altered by the tissue so as to make them ineffective. It is also possible that they are taken up by the cells at a rate which exceeds the diffusion from the fluid to the cell surface. Stirring or mixing of the solutions maintains the block or restores it after recovery. The cells remain sensitive to the original blocking concentration. The degree of inactivation varied with different compounds.

(6) Some of the blocking agents were not inactivated, but the sensitivity of cells to these substances decreased with time of exposure. Of two closely related amino acids, one may be inactivated by the tissue while the other may become ineffective owing to desensitization. The mechanisms of these events are obscure.

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THE INTRALAMINAR DISTRIBUTION OF DIPEPTIDASE ACTIVITY IN HUMAN FRONTAL ISOCORTEX*

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QUANTITATIVE information upon the distribution of biochemical components in relation to microscopic anatomy is an important aspect of the biology of the human nervous system and an essential prerequisite for a comparative, quantitative histochemistry of nervous and mental diseases. Unfortunately, it is seldom possible to obtain specimens of relatively normal human brain in a state suitable for biochemical analyses, including those for metabolites and most enzymes.

The use of psychosurgery for the amelioration of mental disease and the relief of intractable pain provided, for a time, an opportunity to excise biopsy specimens of human cerebral cortex satisfactory for certain types of biochemical analysis. For investigations on this sort of material, the methods of quantitative histochemistry are particularly appropriate. Such techniques enable simultaneous analysis of the biochemical architecture of a tissue such as human cerebral cortex and the acquisition of precise neurochemical information for eventual comparison with clinical data.

Investigations utilizing biopsy specimens of human cortex extirpated during frontal lobotomy were carried out in this laboratory for several years. For the most part, they consisted of studies on the quantitative intralaminar distributions of certain enzymes selected as indices of the location and turnover rates of intracellular metabolic systems critical for the general or special metabolism of brain. The methods used for sampling the cortical layers and sublayers, for quantitative microchemical measurements of enzyme activities, and for correlating the results with cortical anatomical fine structure have represented adaptations to brain of the principles and techniques of quantitive histochemistry originally devised and developed by LINDERSTRØM-LANG (1939) and HOLTER (1951).

The nature, rationale and results of experiments upon the activity and intralaminar distribution of acetylcholinesterase in the frontal cortex of psychotic and non-psychotic patients have been presented previously (POPE, CAVENESS and LIVINGSTON, 1952). Subsequent, analogous studies on proteolytic and oxidative enzymes have also been the subjects of partial, preliminary reports (POPE and HESS, 1954; POPE, HESS and ALLEN, 1957). In this article, experiments on the architectonic distribution of L-alanylglycine dipeptidase activity are described in detail. Only the quantitative histochemical aspects are considered herewith. Possible correlations between the results and certain clinical descriptive parameters will be presented in a separate communication.

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The exact role of intracellular proteolytic enzymes in tissue metabolism is unknown. The fact that tissue peptidases can catalyse the hydrolysis of amide linkages in peptide chains having requisite configurations of amino acid residues is merely a restatement of their operational definitions. Given suitable intracellular conditions (low pH, high reduction potential, presence of specific activators, etc.), such reactions certainly must occur during the life of a cell as well as during post-mortem autolysis. Recent studies on the ability of proteolytic enzymes to perform transamidation and transpeptidation reactions indicate that such enzymes can catalyse steps in the lengthening as well as shortening of peptide chains (JOHNSTON, MYCEK and FRUTON, 1950*a*, *b*). It is also possible that, given suitable thermodynamic conditions, they may catalyse the formation of peptide bonds by a reversal of their more usual hydrolytic role (BERGMANN and FRUTON, 1941). In any case, the relative activities of tissue peptidases can probably be regarded as roughly indicative of rates of tissue protein turnover. In general, tissue proteolytic activity correlates well with other indices of active protein metabolism such as cell density, cellular growth rate and synthetic potentialities, and concentrations of the pentose nucleic acid (PNA) protein complexes that serve as templates for protein synthesis. Cytologically, the PNA-protein is primarily reflected by cytoplasmic basophilia and, in the central nervous system, is roughly coextensive with the characteristic Nissl bodies of the neurones.

The capacity of glycerol extracts of tissues to hydrolyze L-alanylglycine has been rather generally used as a convenient and representative assay system for intracellular proteolytic activity. Earlier observations on the quantitative histochemical distribution of such L-alanylglycine dipeptidase activity in the central nervous system of the albino rat indicated a parallelism with the total cellularity of the part (POPE and ANFINSEN, 1948). A more detailed examination of its intralaminar distribution in rat cerebral cortex (POPE, 1952) showed its activity to be highest in layers II, IV, Vb and VIa. The fact that these are levels relatively rich in nerve cell bodies suggests that neuronal perikarya are the principal intracortical sites of dipeptidase activity. However, activity was well sustained both in the almost acellular plexiform layer (I) and in subcortical white matter. These and other considerations have led to the conclusion that, in rat brain, peptidase activity is related not to neurone population alone, but to that of all cellular species combined. In all probability, proteolytic acitivity resides primarily in the perikarya of both neurones and glia and reflects a high potentiality for protein turnover at these sites. Such a conclusion is also consistent with well-established correlations between this enzyme and the cellular density in many other biological systems (HOLTER and LINDERSTRØM-LANG, 1951). The experiments described herewith that demonstrate the intralaminar profile of L-alanylglycinase in human cortex, indicate that a similar conclusion is probably also appropriate for the brain of man.

MATERIAL AND METHODS

Biopsy specimens of human cortex were excised during the initial stages of prefrontal lobotomy performed according to the open technique described by POPPEN (1948)*. A major portion of the presenting gyrus was isolated and removed atraumatically according to a procedure previously

* All the patients were lobotomized for various forms of mental illness at the Massachusetts Mental Health Center (Boston Psychopathic Hospital). The author is deeply indebted to Dr. H. SOLOMON, Director, and Dr. M. GREENBLATT, Assistant Superintendent and Director of Research and Laboratories at the Massachusetts Mental Health Center, and to Dr. J. POPPEN and associates of the Lahey Clinic Neurosurgical Service for their invaluable co-operation and assistance in making available the cortical biopsy specimens.

described (POPE *et al.*, 1952). It was immediately placed in a refrigerated container and transported without delay to the McLean Hospital Research Laboratory. At once, a block of cortex that included subcortical white matter, and had a relatively flat presenting pial surface and uniform cortical thickness, was dissected from the specimen and frozen with dry ice at −60°C. The remainder of each specimen was used for neuropathological observations which will be described in a subsequent report. The locus of origin and the cyto- and myeloarchitectonic structures of the specimens all were consistent with derivation from the cortical area designated variously as 9 by BRODMANN, as FD_m by VON ECONOMO, and as IE_{fa} by BAILEY and VON BONIN (1951). Architectonically, each consisted of homotypical, eugranular, eulaminate isocortex.

The procedures for preparation of the frozen cortical block and analysis of enzyme activities at uniformly spaced intervals through the architectonic layers and sublayers, and for correlating the results with the anatomical fine structure have all been described in detail previously (POPE, 1952; POPE *et al.*, 1952), and were used without essential change in this series of experiments. A cylinder of cortex, 2 mm in diameter, whose long axis was perpendicular to the plane of the pial surface, was punched from the frozen block and cut with a rotary microtome at −12° into serial, uniform, horizontal, 40 μ thick sections from pia to subcortical white matter. These were used in consecutive groups of four sections each, the first for a histological (Nissl) preparation, the second and fourth for dipeptidase assay and an autolysis control sample respectively, and the third for desiccation and weighing on a quartz fibre torsion balance having a sensitivity of $\pm 0{\cdot}1$ μg. Dipeptidase assay was according to the microtitrimetric method of LINDERSTRØM-LANG and HOLTER (1935) exactly as previously employed for rat cortex (POPE, 1952). The sections for analysis were extracted overnight at 4° in 30% glycerol buffered with 0·67 M-phosphate at pH 7·0. They were then incubated for 30 min at 37° in the presence of 0·1 M-DL-alanylglycine. Freed carboxylate ions were determined by titration in acetone with 0·05 N-alcoholic HCl to the orange end point of naphthyl red. Results were expressed in terms of titration values in 0·05 N-HCl equivalent to the peptide bonds split enzymatically during 30 min hydrolysis per μg dry weight of the adjacent section in the series.

The first section for dipeptidase assay theoretically centred at a depth of 100 μ below the pial surface, and the others at consecutive 160 μ intervals beneath. Accordingly, for each experiment, the observed dipeptidase activities at the levels in question were recorded and plotted in relation to their intracortical loci. Correlation with details of cortical anatomy was accomplished by procedures also previously described in full (POPE, 1952; POPE *et al.*, 1952). Briefly, the enzyme distribution pattern was related to the cytoarchitecture by identification of the architectonic layer of the horizontal histological preparation adjacent to each analysed sample, and comparison with actual measurements of the layer widths in vertical sections cut from the parent cortical block immediately adjacent to the site of the experimental cylinder and impregnated by the Hortega silver method for axis cylinders and neurofibrils. This enabled estimation of the widths of the architectonic layers and sublayers in the experimental cylinder and, in turn, determination of the intracortical distribution of dipeptidase activity in relation to the cytoarchitecture.

For each biopsy specimen, paraffin embedded blocks of cortex were stained by the buffered thionin method of WINDLE, RHINES and RANKIN (1943). In four of these, differential counts of the numbers of neurones, astrocytes, oligodendrocytes, and microglial cells were made at nine levels of the cortex (layers I, II, IIIa, IIIb, IIIc, IV, V, VIa, VIb) and in subjacent white matter. This provided a rough estimate of the relative proportions of the principal cell types at cytoarchitectonically distinguishable and approximately evenly spaced intervals through the cortex and into the subcortical white matter*.

RESULTS

The results of experiments upon the intralaminar distribution of dipeptidase activity in eight different biopsy specimens of human frontal cortex are presented in the accompanying tables and figure. In Table 1, the measured activities at standard depths within the cortex and subjacent white matter are recorded for each experiment in relation to the cytoarchitectonic layers from which each analysed sample was derived. In the last column, the average values for all samples at a given distance beneath the

* The author is indebted to Mrs. MARIE ROSE for preparation of all of the histological material used in this study.

TABLE 1.—DIPEPTIDASE ACTIVITY IN RELATION TO CORTICAL CYTOARCHITECTONIC LAMINATION

Series	Depth (μ)	Layer	Activity (μl 0·05 N-HCl per μg dry wt.)								
			Spec. 1	Spec. 2	Spec. 3	Spec. 4	Spec. 5	Spec. 6	Spec. 7	Spec. 8	Mean
1	100	I	0·32	0·35	—	0·28	—	0·45	0·33	—	0·35
2	260	II	0·32	0·27	0·33	0·25	0·34	0·35	0·22	—	0·30
3	420		0·26	0·27	0·31	0·20	0·27	0·29	0·23	0·24	0·26
4	580		0·24	0·23	0·30	0·25	0·35	0·32	0·23	0·25	0·27
5	740		0·33	0·26	0·29	0·20	0·31	0·30	0·23	0·20	0·27
		III									
6	900		0·31	0·25	0·29	0·27	0·26	0·29	0·24	0·29	0·28
7	1060		0·25	0·25	0·31	0·16	0·23	0·27	0·21	0·29	0·25
8	1220		0·25	0·26	0·26	0·20	0·29	0·27	0·21	0·28	0·25
9	1380		0·31	0·26	0·37	0·27	0·26	0·23	0·23	0·27	0·28
		IV									
10	1540		0·25	0·29	0·28	0·30	0·32	—	0·24	0·28	0·28
11	1700		0·36	0·26	0·24	0·17	0·32	0·29	0·24	0·25	0·27
		V									
12	1860		0·27	0·28	0·35	0·29	0·32	0·27	0·25	0·29	0·29
13	2020		0·25	0·23	0·30	0·28	0·33	0·31	0·28	0 28	0·28
14	2180		0·29	0·25	0·27	0·22	0·28	0·29	0·24	0·26	0·26
15	2340		0·25	0·29	0·22	0·22	0·26	0·28	0·24	0·26	0·25
		VI									
16	2500		0·24	0·23	0·23	0·27	0·30	0·32	0·25	0·26	0·26
17	2660		0·25	0·15	—	0·28	0·30	0·30	0·29	0·26	0·26
18	2820		0·27	—	—	0·19	0·33	0·28	0·23	0·26	0·26
19	2980		0·23	—	—	0·25	0·34	0·28	0·27	0·27	0·27
20	3140	WM	0·25	—	—	0·26	0·32	0·28	0·26	0·25	0·27
21	3300		0·27	—	—	0·28	0·32	—	0·20	0·25	0·26
Mean, cortex only			0·28	0·26	0·26	0·29	0·29	0·30	0·24	0·26	0·27

pial surface are similarly related to the average widths of the layers in this series of experiments. Also included for each specimen is the dipeptidase activity of the cortex as a whole (mean values for layers I–VIb).

In Fig. 1, the results in the final column of Table 1 are presented graphically. The average dipeptidase activity is plotted as a function of the intracortical depth of the serial samples and in relation to the average cytoarchitectonic layer widths. The result is, therefore, a profile representing the cytoarchitectonic distribution pattern of L-alanylglycine dipeptidase in human frontal isocortex.

In Table 2, the means of the average activity per specimen of all samples assignable to a given cytoarchitectonic layer or sublayer are presented together with the standard errors of these means. The averages of the relative differential cell counts made at

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comparable intracortical levels and of the widths of the architectonic layers in this series of experiments are also shown.

The results in Table 1 and the graphical representation of their means (Fig. 1)

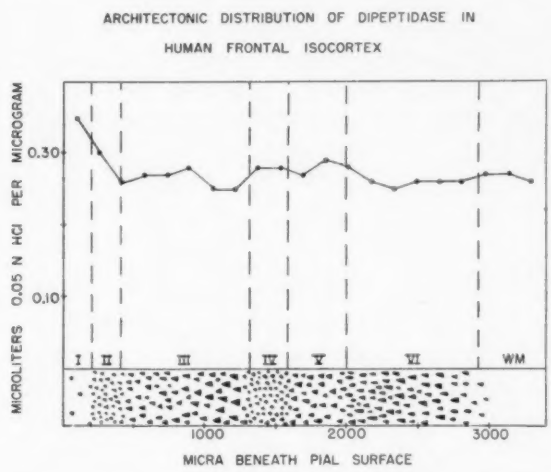

FIG. 1.—Distribution pattern of dipeptidase activity in relation to cellular lamination of human frontal isocortex. The sketch at the bottom of the figure shows the characteristic cytoarchitectonic structure with respect to the differential size, shape and density of the nerve cell bodies within the constituent cortical layers and sublayers. The cytoarchitecture of the cortical biopsy specimens used for this study corresponded exactly with the description of 'typical eulaminate isocortex' by BAILEY and VON BONIN (1951) and in detail corresponded with their block VI derived from the superior frontal convolution and designated area IE_{f_2}.

TABLE 2.—DIPEPTIDASE ACTIVITY IN RELATION TO CORTICAL CELL TYPES

Layer width (μ)		Pept.*	S.E.M.	No. cells per oil immersion field Neur.	Astros.	Oligos.	Micros.	Total
I	200	0·35	0·03	3	8	2	2	15
II	210	0·29	0·02	19	4	5	1	29
IIIa	920	0·27	0·01	9	3	3	2	17
b		0·27	0·01	9	4	4	2	19
c		0·26	0·01	10	7	4	2	23
IV	250	0·27	0·01	18	5	5	2	30
V	420	0·29	0·01	7	4	3	3	17
VIa	930	0·27	0·01	12	5	4	2	23
b		0·26	0·01	6	7	7	1	21
WM		0·25	0·02	0	7	20	2	29

* Expressed as titration value in μl 0·05 N-HCl per μg dry weight. Each value is the average, for all eight experiments, of the means in the individual experiments of all samples derived from the designated cytoarchitectonic layers and sublayers.

provide information that depends solely on the measured intracortical depth of the analysed samples and upon which judgement concerning the histological structure is merely superimposed. On the other hand, the method of presentation in Table 2 is

dependent upon the classical and familiar neuroanatomical analysis of cortical structure in terms of its cytoarchitecture. It yields information concerning the biological variability of depeptidase activity in the commonly recognized cytoarchitectonic layers and sublayers in the frontal cortex of man.*

Whichever way the results are viewed, essentially the same features of the intracortical distribution of L-alanylglycine dipeptidase are brought out. In spite of minor fluctuations in activity from level to level, the intralaminar profile of the enzyme is chiefly remarkable for its uniformity and for the absence of major differences in proteolytic activity within the different cortical layers and sublayers. With the exception of layer I, the maximum deviations from the cortical mean of single observations were never more than 33 per cent of the value of the mean and, in most instances, they were very much less.

The plexiform layer (I) contrasts somewhat with the rest of the cortex. In each of the five experiments in which layer I was successfully sampled, its dipeptidase activity was greater than the mean for the cortex as a whole and, in three instances, the increment was considerable. In four of seven experiments, the activity was also relatively high in layer II, and similar minor elevations occurred in layer IIIb (seven of eight experiments), IV (five of seven experiments), and V (seven of eight experiments). On the other hand, activity in layer IIIc was somewhat lower than that of the cortical mean, or of immediately adjacent zones, in seven of eight experiments. In five of the six instances in which subjacent white matter was sampled, its activity was essentially the same as that of the grey cortex.

The differential cell counts at ten cytoarchitectonic levels (Table 2) showed the predictable increases in neurone population in the granular layers (II, IV) and upper half of the fusiform cell layer (VIa). The glia, as a whole, were more uniformly distributed. Astrocytes were relatively abundant in layer I and in the lower third of layer III corresponding, respectively, with the zones of subpial cells and of highest capillary density. They also became somewhat more numerous as the white matter was approached (VIb) and entered. As might be expected, oligodendrocytes followed roughly the neurone population, but increased in layer VI, due both to the relative abundance of myelinated fibres and the characteristically larger number of perineuronal satellites in this layer. Oligodendrocytes became exceedingly numerous in the white matter where they constitute the dominant cell type. Resting microglial cells were the least abundant cell type and showed no tendency towards lamination.

In summary, the results show that dipeptidase activity is essentially constant within the layers and sublayers of human frontal cortex and in the subjacent white matter. A single exception is the relatively high activity observed in the plexiform layer. Otherwise, the lack of definite or consistent differences in dipeptidase activity at different intracortical levels contrasts with the cytoarchitectonic distribution pattern of the same enzyme in rat somatosensory isocortex (POPE, 1952) and, in general, with those of other enzymes in rat (POPE, 1952; POPE, HESS, WARE and THOMSON, 1956; KUHLMAN and LOWRY, 1956), rabbit (LOWRY, ROBERTS, WU, HIXON and CRAWFORD, 1954), monkey (ROBINS, SMITH, EDYT and MCCAMAN, 1956), and human cortex (POPE *et al.*, 1952, 1957). Another noteworthy feature is the sustained dipeptidase activity

* The problem of how best to present and analyse the results of experiments of this type, the advantages and disadvantages of each form of presentation herewith employed, and the significance of statistical treatment of data of this sort, all have been discussed at length in a recent communication from this laboratory (HESS and POPE, in press).

in subcortical white matter where, if recalculated on the basis of an estimated fat-free dry weight (or total protein), it was actually higher than in the cortical grey matter. In this respect also, dipeptidase seems to be unusual, since most intracellular enzymes are more active in grey matter than in white. Finally, dipeptidase activity in human cortex and subcortical white matter was increased two or threefold over that found previously for various portions of rat nervous system (POPE and ANFINSEN, 1948), including the cerebral cortex (POPE, 1952). This is in sharp contrast to the relationship between rats and man in the case of other enzymes studied in a comparable way and to the generally inverse relationship between mammalian metabolic rates and body weight.

DISCUSSION

The results of these experiments will be discussed in terms of their anatomical significance and their relationship to the biochemistry of the cerebral cortex.

In previously reported studies, certain correlations have been established between the non-uniform intracortical distributions of biochemical substances and the corresponding lamination of histological components (POPE, 1952; POPE *et al.*, 1952, 1956). Paradoxically, the very simplicity and uniformity of the intracortical dipeptidase profile in man makes establishment of correlations between it and the histological structure exceedingly difficult. The intra-laminar distribution of dipeptidase bears no simple relationship to any single structural element or group of elements. It conforms neither to the cellular nor vascular architecture nor to the distribution of nerve cells, astrocytes, oligodendrocytes or microglial cells, or of those of the cortical plexuses of axones and dendrites, in so far as these are accurately known in this region of human cortex.

In rat cortex, the architectonic distribution of dipeptidase activity qualitatively paralleled the total number of cell bodies, both neuronal and glial, in a standard cortical volume (POPE, 1952). Moreover, calculations have shown that the mean-dipeptidase acitivities per unit fat-free dry weight in the cortex as a whole and in the subjacent white matter were essentially the same.* So, also, were the average total number of cells in equivalent volumes of cortex and white matter. Thus, the dipeptidase : cell ratio appeared to be approximately constant, irrespective of the nature of the cell population. Accordingly, the results have been regarded as consistent with localization of proteolytic activity in the cell bodies of both neurones and neuroglia. Although less readily apparent, an analogous relationship probably exists in the cortex of man. However, careful comparison of the dipeptidase distribution with the microscopic anatomy leads to the conclusion that proteolytic activity is directly related not to the total number of cellular units, but rather to the total mass of neuronal and glial protoplasm. The considerations leading to this conclusion are described below.

The results presented in Tables 1 and 2 show that, per unit dry weight, dipeptidase activity is essentially the same in human subcortical white matter as it is (on the average) in the grey cortex. Since, however, the metabolically inert myelin lipids contribute relatively so large a fraction of the dry weight of white matter, it is useful to recalculate the enzyme activities in terms of an estimated fat-free dry weight.* For practical

* The fat-free dry weights of the samples of rat cortex referred to (POPE, 1952) and of human cortex used in this study were estimated on the assumption that 35 per cent of the dry weight of grey matter and 60 per cent of that of white matter is contributed by the lipids. Actual extraction of similar sections of human cortex and white matter with lipid solvents results in weight losses of exactly these orders of magnitude.

purposes, the latter is roughly equivalent to the mass of actively metabolizing tissue. If this is done with the foregoing data, dipeptidase proves to be approximately 1·6 times more active in the subjacent white matter than in the cortex as a whole. Results of the differential cell counts (Table 2) show that the total number of cells in a standard volume is also greater in white matter as compared with cortical grey and by approximately the same ratio (1·4 in this instance). As a first approximation, therefore, in the human as in the rat, brain proteolytic activity appears to be dependent upon the total number of cells in the sample, but to be independent of whether these cells are almost exclusively neuroglia (as in white matter) or a mixture of neurones and glia.

Closer scrutiny of the experimental results shows that within the cortex proper the relationship between dipeptidase activity and cell population is not so simple or direct. The plexiform layer is obviously at variance with any such interpretation and will be discussed further below. Even with the exclusion of layer I, it is easily apparent that the distribution of dipeptidase does not parallel the nerve cell population. Clearly, this statement is merely a reassertion of the uniformity of intracortical proteolytic activity, inasmuch as the nerve cell population shows the classical non-uniform distribution of eulaminate isocortex. It appears, therefore, that the proteolytic activity per neurone is relatively low in the granular layers (II and IV) and relatively high in layers III and V. Because of the differences in cell size, however, the amounts of neuronal perikaryon material may be expected to show a similar relationship. Indeed, the quantitative anatomical studies of S. T. BOK (1936) on human temporal lobe cortex led this author to conclude that the volume of neuronal perikaryon material is constant at all levels from layer II through layer VI inclusive. The constancy of intracortical dipeptidase distribution would accord well with this proposition if proteolytic activity in brain is, in some measure, localized in the perikarya of the nerve cells. That it cannot be exclusively so is shown by the high activity in layer I and in subjacent white matter. This leads to consideration of the role of glial elements in determining dipeptidase distribution in human central nervous system.

Within the cortex itself, the neuroglia show little tendency towards lamination, and consequently, intracortical dipeptidase activity parallels better the total number of glial cells per unit volume than it does the number of neurones or that of all cell species combined. A number of observations have suggested that neuroglia, particularly astrocytes, may be important sites of proteolytic activity in the brain of man. One is the relative abundance of such activity in human as compared with rat brain. In man, both the relative number of neuroglial cells and the mass and degree of arborization of their processes are considerably greater than in lower animals. In addition, the high activities in white matter and in layer I occur at levels where neuroglia contribute a major part of the volume fraction of the tissue. Finally, dipeptidase activity is high in regions of gliosis and in tumours of the glioma group, particularly astrocytomas (POPE *et al.*, 1957). Closer scrutiny of these facts, however, shows that quantitatively the apparent correlations tend to break down. While the glia are more abundant in human than in rat brain, it is not by a factor of seven which is the ratio of dipeptidase activity per cell between human and rat cortex. Moreover, while dipeptidase is high in gliomas, the activity per cell is lower than it is in normal brain. Also, in tabetic dorsal root ganglia, dipeptidase activity decreases in direct proportion to the diminution in number of neurones (CAVENESS, ADAMS, POPE and WEGNER, 1949). Finally, while the dipeptidase: total glial cell ratio is fairly constant in the cortical layers, it

becomes very much reduced in subjacent white matter because of the threefold increase in neuroglial population as compared with only a 50 per cent increase in dipeptidase activity, even if the latter is estimated on a defatted dry weight basis. All the foregoing observations indicate that glial elements contribute importantly to the proteolytic activity of human cortex, but cannot be solely responsible for it. The conclusion seems inescapable that the neurones are also an important, if not the chief source of such activity in grey matter.

Further discussion of the problem presented by the plexiform layer is in order. In rat cortex, dipeptidase activity was lower in layer I than in layer II or in the cortex as a whole, whereas the reverse is true in the case of man. This apparent discrepancy can be reconciled if the dipeptidase activities in layer I of the two species are compared with the respective cell populations. In both instances, the ratio of activity to the total number of cells present (chiefly glia) is about double that found in all the other cortical layers. This means that some element in the plexiform layer in both species provides an increment of proteolytic activity not found in the rest of the cortex or its subjacent white matter. The unique element at this level responsible for this phenomenon may well be the pia mater, including its fibrous tissue and blood vessels. Note should also be made of the fact that the neuronal protoplasmic mass as compared with number of nerve cell bodies is abnormally high in the plexiform layer, owing to the presence of the terminal bushels of apical dendrites derived from cortical pyramids and spindle cells whose cell bodies are situated in all the deeper layers.

Thus, in human cortex, there is little or no evidence for selective localization of proteolytic activity in any one type of cell or portion thereof. The only rational conclusion consistent with the facts cited is that dipeptidase activity is present in all cortical cell species including both neurones and the several types of glia. Moreover, it would appear to be related only very approximately to the total number of cells. From the characteristics of the intralaminar dipeptidase profile, the most logical inference seems to be that the enzyme resides in the perikarya and protoplasmic processes of the various cell types and is, therefore, a reflection more of the amount of actively metabolizing protoplasmic mass than of the number of cellular units.

If this interpretation is correct, it means that proteolytic activity in human brain is distributed in relation to anatomical components in a manner similar to that found previously for the central nervous system of the rat (POPE and ANFINSEN, 1948; POPE, 1952). In both instances, the localization is analogous to that of a number of proteolytic enzymes in a wide variety of biological systems (HOLTER and LINDERSTRØM-LANG, 1951). In many organs and tissues of both vertebrate and invertebrate animals, peptidases and proteinases have repeatedly been shown to reflect either the cell population or mass of actively metabolizing protoplasm. The results of the experiments reported herewith, as well as those of previously reported studies on rat brain, indicate that a similar situation exists in the mammalian nervous system including that of man.

While the biological role of intracellular peptidases remains obscure, measurement of their comparative activity rates has, as indicated above, been widely used as a means of estimating rates of tissue protein turnover. Assuming this to be a valid principle, it means that human cerebral cortex and white matter have a very high potential for protein metabolism and that this resides within each one of the major histological components of these structures. Many previous lines of work have emphasized the

capacity of nerve cell bodies to synthesize protein which, in all probability, is then transported to all parts of the neural apparatus. Protein synthesis almost certainly occurs with the PNA of the basophilic Nissl substance serving as template. A correspondingly high potentiality for the breakdown and rearrangement of peptide chains would accord well with this synthetic potential. The capacity of neuroglia for protein synthesis has never been carefully investigated. The fact that such cells do possess active mechanisms for protein breakdown suggests that they too are importantly involved in the formation of structural chemical components in brain. Such synthetic ability would be consistent with the well known capacity of astrocytes for hyperplasia and, perhaps, also with the satellite and myelin formation roles of the oligodendrocytes.

In conclusion, the remarkably high proteolytic potential of human brain should be stressed once more. Alanylglycine dipeptidase activity is almost three times as great in human as in rat brain if expressed on a gravimetric basis, and if the phylogenetic decrease in cell density is taken into consideration, the proteolytic activity per cell is approximately sixfold. It is possible that this capacity for rapid protein turnover is a reflection of the relatively long protoplasmic and especially fibrillar processes of nerve cells in the human nervous system with the consequent enhancement of the maintenance problem for structural components. To a lesser extent, similar considerations might apply to the neuroglia of man which by and large show a greater elaboration of their protoplasmic expansions than do those of lower animals. In any event, the problem of protein turnover in the brain of man is one with many important ramifications with respect to both the biochemical economy of the normal organ and to the possible implications it may have for understanding of the genesis of many neurological diseases.

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SUMMARY

By means of microchemical techniques, the activity and intralaminar distribution of L-alanylglycine dipeptidase have been determined in biopsy specimens of human frontal cortex.

Dipeptidase activity is high in human cortex. This indicates a considerable proteolytic potential and presumably a high rate of protein turnover in the brain of man.

The cytoarchitectonic distribution pattern of dipeptidase activity shows it to be higher in layer I than in layers II to VI in which it is essentially constant. The activity (on a dry weight basis) in subcortical white matter is the same as the average for cortical grey matter.

Analysis of these results in terms of cortical histological structure shows them to be consistent with localization of cortical proteolytic activity in the perikarya and protoplasmic expansions of both neurones and glial cells.

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THE METABOLISM OF CHOLESTEROL AND FATTY ACIDS IN THE CENTRAL NERVOUS SYSTEM*

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UNIFORMLY negative results have been obtained in attempts to incorporate deuterium-labelled or ^{14}C-labelled acetic acid into cholesterol of the adult mammalian brain (SRERE, CHAIKOFF, TREITMAN and BURSTEIN, 1950; BLOCK, 1950; AZARNOFF, CURRAN and WILLIAMSON, 1957). In addition to these data, no turnover was detected in the non-saponifiable fraction of adult rat brain after administering heavy water to rats, although fatty acid synthesis was detected under these circumstances (WAELSCH, SPERRY and STOYANOFF, 1940). The following experiments were performed to investigate the ability of the central nervous system to incorporate ^{14}C-labelled acetic acid into cholesterol and fatty acids upon observing that considerable ^{14}C was incorporated into brain cholesterol by injecting sodium [α-^{14}C] acetate directly into this organ.

MATERIALS AND METHODS

Young female rats (30–50 days old, 90–150 g in weight) and adult female rats (6 months and 1 year old, weighing 190–250 and 250–350 g respectively) were used for most of these studies. All animals were sacrificed by decapitation. The sodium [α-^{14}C] acetate was purchased from the Isotopes Specialties Company, Burbank, California, and had a specific activity of 2·6 mC/m-mole.

In vivo procedures. Rats were injected intraperitoneally with an aqueous solution of sodium [α-^{14}C] acetate. Before alkaline digestion, excised organs were rinsed several times in distilled water. The brain consisted essentially of cerebral hemispheres, cerebellum and most of the medulla. The spinal cord consisted of that portion from the severed end to the lumbar region.

Operative techniques: intracerebral injection. Young rats were subjected to light ether anaesthesia, whereupon a no. 26 hypodermic needle was pushed directly through the skull into the selected area to a depth of about 0·8 cm. It was not difficult to control the depth of injection, which was always directed towards the centre of the brain substance. Although occasional leakage was observed at the injection site, no plugging of the wound was performed. For adult rats the procedure used by HESS to produce uniform stab wounds was found satisfactory (HESS, 1955). Under deep ether anaesthesia the hair was shaved and an incision made through the skin. Muscle and periosteum overlying the skull above the right cerebral hemisphere were cut off and a small hole was made in the skull with a dental drill. The operated area was swabbed with alcohol and allowed to heal without further treatment. From 3 to 10 days later a no. 26 needle was used to inject the desired quantity of labeled sodium acetate solution, this time under light ether anaesthesia. With the needle still inserted, a drop of collodion was placed over the injection area, after which the needle was withdrawn. In young animals the volume of acetate solution was kept to 0·05 ml. In adult animals this was raised to 0·1 ml

Evisceration and hepatectomy. A group of 3 month old female rats was subjected to the procedure described by SRERE *et al.* (1950), except that after ligation of the portal vein the liver lobes were completely severed.† Two intramuscular injections of 500 mg each of glucose were given during

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† We are indebted to Dr. C. BLY for performing these operations.

the operation. Only two animals survived the operation sufficiently long to warrant intracerebral injection of labelled acetate. Within a few minutes after removal of the last bit of liver tissue, $4{\cdot}4 \times 10^6$ counts/min of sodium [α-^{14}C] acetate was injected directly into the right cerebral hemisphere in the manner previously described. One rat was sacrificed 69 min after injection and the other, in very poor condition, 87 min after injection.

Chemical procedures. For digestion, tissues were heated on the steam bath for several hours with a mixture containing 50% ethanol (v/v) and 15% KOH (w/v). The non-saponifiable matter was extracted by five portions of petroleum ether (b.p. 30-60°). The combined extracts were extracted once with 5% aqueous KOH, washed three times with water and distilled. Last traces of liquid were removed under nitrogen on the steam bath. The residue was dissolved in acetone–ethanol (1 : 1, v/v), centrifuged to remove some insoluble matter, and cholesterol as the digitonide was isolated by the Sperry–Webb method (SPERRY and WEBB, 1950). The digitonides were suspended in ether and filtered onto 3 cm² pieces of Whatman No. 1 filter paper. The uniformly distributed digitonides on the paper were placed in nickel planchets and counted for ^{14}C-activity. Sufficient counts were taken to give a standard deviation of 5 per cent or less. Corrections were made to infinitely thick samples using a correction curve experimentally established for the counting instrument. Two digitonides from the adult brains following intracerebral injection were converted to free cholesterol (FRAME, 1944) and then to the dibromide (SCHWENK and WERTHESSEN, 1952). Following precipitation of cholesterol as the digitonide from the non-saponifiable material, the supernatant fluid (and washes) were evaporated to low volume under N_2 on the steam bath and rediluted with acetone–ethanol (1 : 1, v/v). Additional digitonin solution was added to insure precipitation of all cholesterol. After standing sealed overnight, the mixture was centrifuged. Portions were plated from the supernatant fluid on planchets, dried, and counted as non-digitonin precipitable ('NDP') fractions in infinitely thin samples.

The original aqueous phase and KOH extract were combined and extracted once with petroleum ether. The petroleum ether phase was discarded. The aqueous alkaline phase was then acidified

TABLE 1.—INCORPORATION OF INTRAPERITONEALLY INJECTED SODIUM [α-^{14}C] ACETATE INTO CHOLESTEROL OF RATS OF VARIOUS AGES*

Rat no.	Age	Time sacrificed after injection (hr)	Cholesterol specific activity (counts/min per mg)		
			Brain	Liver	Sp. cord
1	35 days	3	23	251	—
2		3	27	228	—
3		6	36	427	—
4		6	32	283	—
5		16	26	78	—
6		16	23	133	—
7	6 months	3	1·7	101	—
8		3	1·8	86	—
9		6	1·8	62	—
10		6	1·9	49	—
11		16	3·5	159	—
12		16	2·4	91	—
13	1 year	3	0·7	26	0·5
14		3	0·8	96	0·4
15		3	0·6	39	0·4

* Rats 1–12 received $4{\cdot}4 \times 10^7$ counts/min sodium [α-^{14}C] acetate. Rats 13–15 received $2{\cdot}6 \times 10^7$ counts/min sodium [α-^{14}C] acetate.

with HCl and extracted with one portion of petroleum ether. The latter was washed three times with distilled water to give a crude fatty acid fraction. This was dissolved in benzene and portions were plated and counted as infinitely thin samples. The cholesterol specific activity given in the tables was calculated from the digitonide values as infinitely thick samples.

All ^{14}C determinations were made with a windowless gas flow counter.

RESULTS AND DISCUSSION

When sodium [α-^{14}C] acetate was injected intraperitoneally, a progressive decrease with age in incorporation of ^{14}C into central nervous system cholesterol was found (Table 1). This parallels previously published data showing the same progressive decrease in synthetic capacity with age (up to 40 days), but utilizing deuterium incorporation into cholesterol from heavy water injected subcutaneously (WAELSCH, SPERRY and STOYANOFF, 1941). Animals sacrificed at various time intervals following injection showed no consistent increase in ^{14}C incorporation. The specific activity of brain fatty acids following intraperitoneal injection of sodium [α-^{14}C] acetate was

TABLE 2.—INCORPORATION OF INTRACEREBRALLY INJECTED SODIUM [α-^{14}C] ACETATE INTO CHOLESTEROL OF YOUNG AND ADULT RATS*

Rat no.	Time sacrificed after injection	Age	Cholesterol specific activity (counts/min per mg)		
			Brain	Liver	Sp. cord
1	3 hr	35 days	85	13	—
2	3 hr	35 days	59	26	—
3	9 hr	35 days	87	8	—
4	9 hr	35 days	92	7	—
5	24 hr	35 days	60	5	—
6	24 hr	35 days	44	5	—
7	24 hr	32 days	85	—	—
8	24 hr	32 days	48	—	—
9	6 days	32 days	56	0	—
10	6 days	32 days	65	0	—
11	12 days	32 days	70	0	—
12	12 days	32 days	74	0	—
13	4 weeks	32 days	69	0	81
14	4 weeks	32 days	107	0	112
15	3 hr	1 year	16	17	19
16	3 hr	1 year	15	18	0
17	3 hr	1 year	0	23	0
18	3 hr	1 year	13	4	10
19	20 hr	1 year	20	9	20
20	20 hr	1 year	41	11	13
21	20 hr	1 year	60	15	29
22	20 hr	1 year	25	15	0
23	3 hr	1 year	45	7	—
24	3 hr	1 year	17	6	—
25	3 hr	1 year	20	24	—
26	3 hr	1 year	0	20	—

* Rats 1–14 received $4{\cdot}4 \times 10^6$ counts/min sodium [α-^{14}C] acetate. Rats 15–17 received $1{\cdot}3 \times 10^7$ counts/min sodium [α-^{14}C] acetate. Rats 18–26 received $2{\cdot}6 \times 10^7$ counts/min sodium [α-^{14}C] acetate.

about equal to fatty acid specific activity obtained on *in vitro* incubation of brain minces (see Table 5).

A marked increase in sodium [α-^{14}C] acetate incorporation into central nervous system cholesterol was shown on injecting the acetate directly into the brain (Table 2). An arbitrary time period of 3 hr was selected as a standard sacrifice period following injection. Increasing this time period had no consistent effect on increasing or decreasing incorporation of ^{14}C, as shown in Table 2. By extending these studies further, the outstanding metabolic inertness of labelled central nervous system cholesterol soon became apparent (NICHOLAS and THOMAS, 1957). This aspect will be discussed shortly. Tables 3 and 4 show that the incorporation phenomenon is not localized at the injection site. When the cerebral hemispheres were examined separately, both were radioactive (Table 4). Spinal cords of young and adult animals were

TABLE 3.—EFFECT OF INJECTION POSITION ON INCORPORATION OF SODIUM [α-^{14}C] ACETATE INTO CHOLESTEROL OF RATS BY INTRACEREBRAL INJECTION*

Rat no.	Injection position	Cholesterol specific activity (counts/min per mg) Brain	Liver
1	Between eyes—midline	47	13
2	Between eyes—midline	0	—
3	Between eyes—midline	46	21
4	Between eyes—midline	63	—
5	Between eyes—midline	0	—
6	Between ears—midline	109	42
7	Between ears—midline	46	—
8	Between ears—midline	81	19
9	Between ears—midline	73	—
10	Between ears—midline	50	—

* Each rat received 4·4 × 10^6 counts/min sodium [α-^{14}C] acetate. All rats 31 days old; sacrificed 3 hr after injection.

TABLE 4.—INCORPORATION OF SODIUM [α-^{14}C] ACETATE INTO BRAIN CHOLESTEROL AFTER INJECTION INTO OPPOSITE HEMISPHERE*

Rat no.	Brain section	Cholesterol specific activity brain (counts/min per mg)
1A	Left hemisphere—injected	25
1B	Right hemisphere—non-injected	59
2A	Left hemisphere—injected	18
2B	Right hemisphere—non-injected	54
3A	Right hemisphere—injected	7
3B	Left hemisphere—non-injected	6
4A	Right hemisphere—injected	48
4B	Left hemisphere—non-injected	54

* Each rat received 4·4 × 10^6 counts/min sodium [α-^{14}C] acetate. All rats 32 days old; sacrificed 3 hr after injection.

radioactive despite injection into distant parts of the central nervous system (Table 2). Occasional failure to detect ^{14}C in the cholesterol digitonides (Table 2) was attributed to extensive leakage of fluid, including acetate, from the site of injection. MCMILLAN, DOUGLAS and MORTENSON (1957) in a recent interesting manuscript have shown that brain cholesterol (young and adult) can be labelled with ^{14}C by injecting [α-^{14}C] acetate into the cisterna magna and have also pointed out the marked length of time with which brains so injected remained labelled with ^{14}C.

Initially it was thought that the ^{14}C incorporation found in the brain cholesterol after intracerebral injection might in some way be the result of transfer from the liver. This seemed highly improbable because of the difference in specific activities of liver and brain cholesterol. Incorporation into the brain in the absence of liver and viscera (see Experimental) eliminated this possibility. In the two rats which survived the manipulative procedure brain cholesterol specific activities of 0 and 25 were found. We have attributed the negative result to adverse penetration of the needle into the skull with consequent loss of radioactive acetate. Although only one positive result was obtained, the nature of the experiment obviates the cholesterol in this case coming from the liver. Two of the adult brain cholesterol digitonides (from rats 21 and 23

TABLE 5.—INCORPORATION OF ^{14}C INTO ADULT BRAIN FATTY ACIDS FROM SODIUM [α-^{14}C] ACETATE ADMINISTERED BY VARIOUS ROUTES*

Rat no.	Method of administration	Brain fatty acid specific activity (counts/min per mg)
1	Intraperitoneal injection	30
2	Intraperitoneal injection	16
3	Intraperitoneal injection	20
4	Intracerebral injection	333
5	Intracerebral injection	156
6	Intracerebral injection	457
7	Intracerebral injection	14
8	Intracerebral injection	463
9	Intracerebral injection	764
10	Intracerebral injection	1273
11	Intracerebral injection	562
12	Intracerebral injection	746
13	Intracerebral injection	480
14	Intracerebral injection	545
15	Intracerebral injection	810
16	Incubation *in vitro*	27
17	Incubation *in vitro*	48

* Rats 1–3 received $2{\cdot}6 \times 10^7$ counts/min sodium [α-^{14}C] acetate. See Table 1 for cholesterol specific activity from corresponding rat brains (rats 13–15). Rats 4–6 received $1{\cdot}3 \times 10^7$ counts/min and rats 7–15 received $2{\cdot}6 \times 10^7$ counts/min sodium [α-^{14}C] acetate. See Table 2 for cholesterol specific activity from corresponding rat brains (rats 15–25). Fatty acids of flasks 16–17 were obtained from flasks containing phosphate buffer pH 7·4 (10 ml) nicotinamide (5·0 mg), DPN (0·5 mg), ATP (5·0 mg), and glucose (100·0 mg) per approximately 1 g of brain mince. To each flask $2{\cdot}6 \times 10^7$ counts/min sodium [α-^{14}C] acetate was added. Incubation 3 hr at 37° under O_2. All rats were 1 year old.

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of Table 2) were converted through free cholesterol to the dibromides with little loss of radioactivity.

Both cholesterol and fatty acid synthesis (Table 5) were minimal in adult brain *in vitro* and by intraperitoneal injection, yet were markedly increased on intracerebral injection. The synthetic capacity of adult excised brain tissue in systems tested thus far may not, therefore, represent the true synthetic capacity of the adult brain *in situ* for cholesterol or fatty acids.

In order to determine how long the central nervous system may retain appreciable quantities of ^{14}C, a long-range study was instituted, yielding the data in Tables 6 and 7. In short-term experiments brain cholesterol showed higher specific activities

TABLE 6.—METABOLISM OF CHOLESTEROL IN THE CENTRAL NERVOUS SYSTEM*

Rat no.	Time of sacrifice	Cholesterol specific activity (counts/min per mg)	
		Brain	Spinal cord
1	7 weeks after injection	48	103
2	7 weeks after injection	40	144
3	5 months after injection	13	31
4	5 months after injection	8	21
5	8 months after injection	26	80
6	8 months after injection	26	68
7	10 months after injection	35	80
8	10 months aftre injection	37	86
9	12 months after injection	31	59
10	12 months after injection	34	103

* All animals (females) injected at 35 days of age. Each rat received $4{\cdot}4 \times 10^6$ counts/min sodium [α-^{14}C] acetate.

TABLE 7.—THE METABOLISM OF LIPIDES IN THE CENTRAL NERVOUS SYSTEM. LONG-TERM STUDIES*

Rat no.	Tissue	Specific activity cholesterol fatty acids (counts/min per mg)		Non-saponifiable non-digitonin precipitable (NDP) (total counts/min in organ)
1	Brain	31	68	$1{\cdot}87 \times 10^3$
1	Spinal cord	59	81	$9{\cdot}20 \times 10^3$
2	Brain	34	28	$4{\cdot}04 \times 10^3$
2	Spinal cord	103	210	$1{\cdot}59 \times 10^5$

* Each animal injected at age 35 days old with $4{\cdot}4 \times 10^6$ counts/min sodium [α-^{14}C] acetate and sacrificed 1 year later.

than spinal cord cholesterol. As time proceeded this relationship became reversed (Table 6) and is presumably related to the slower rate of turnover of cholesterol in the spinal cord as compared to brain cholesterol. It appears that both brain and spinal cord cholesterol are metabolized at an extremely slow rate and that once ^{14}C is incorporated into central nervous system cholesterol it may remain there indefinitely.

Examination of fatty acids and non-saponifiable material other than cholesterol (referred to as non-digitonin precipitable material or 'NDP' in this and subsequent publications) revealed that this resistance to metabolism was not limited to cholesterol alone (Table 7). In the two brains examined the fatty acid specific activity was much lower than that encountered in short-term experiments, suggesting that turnover may be more rapid for these substances than for cholesterol.

Most of the data presented in this manuscript can best be rationalized on the basis of a functioning 'blood–brain barrier' which prevents acetate from entering the brain tissue readily and becomes progressively more active as the animal matures. Numerous data have established that a blood–brain barrier exists for a large number of molecules; its principal role appears to be that of slowing down the entry of various substances into the brain (BAKAY, 1956). This is not the complete answer, however, or acetate would be incorporated readily into brain cholesterol *in vitro*, where such a barrier does not exist. General experience has shown that *in vitro* incorporation of acetate into adult brain cholesterol is negligible (SRERE, CHAIKOFF, TREITMAN and BURSTEIN, 1950; AZARNOFF, CURRAN and WILLIAMSON, 1957). Our work is in agreement with this, although like ROSSITER we have consistently found slight incorporation of ^{14}C into cholesterol digitonides when labelled acetate is incubated with adult brain tissue (ROSSITER, 1957; NICHOLAS and THOMAS, 1958). Obviously some factor (*s*), available to the brain on needle puncture, is absent when *in vitro* experiments are performed with adult tissue.

In attempting to compare these findings with the earlier and excellent work utilizing deuterium (WAELSCH, SPERRY and STOYANOFF, 1940, 1941) it should be pointed out that the sensitivity of the deuterium method is considerably less than that of the ^{14}C method. Since any synthesis of cholesterol found with the aid of ^{14}C acetate represents only a minute fraction of the total cholesterol present, the ^{14}C data may not necessarily represent a synthetic ability of brain tissue (as regards cholesterol synthesis) greater than previously reported with the use of deuterium. On the other hand, certain discrepancies exist which must be considered in any further evaluation. For example, while the lengthy retention of ^{14}C (as long as a year) is certainly indicative of the slow or negligible turnover previously established for brain non-saponifiable matter, the rapid incorporation of ^{14}C (3 hr or less) on intracerebral injection is not. Deuterium is freely permeable to the blood–brain barrier and its entrance into the brain substance does not necessitate puncturing the brain area in any way. Until the response of the brain to acetate introduced by needle wounds or subarachnoid administration can be assessed more thoroughly, it will probably be best not to compare the two methods in more detail.

SUMMARY

The incorporation of ^{14}C-methyl-labelled sodium acetate into young and adult rat brain cholesterol and fatty acids was accomplished by injecting the acetate

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directly into the brain, thus by-passing the blood–brain barrier. Only small or negligible amounts of ^{14}C were incorporated into these substances when the acetate was injected intraperitoneally into adult rats. On intracerebral injection non-saponifiable material not precipitated by digitonin became labelled also. ^{14}C activity in these various lipide fractions was detected as much as a year after injection.

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ÜBER DIE AUTOXYDATION EMULGIERTER GEHIRNPHOSPHATIDE UND MENSCHLICHER HIRNGEWEBSHOMOGENATE UND DEN NACHWEIS DABEI GEBILDETER PEROXYDE

THE AUTOXIDATION OF EMULSIFIED BRAIN PHOSPHATIDES AND OF PHOSPHATIDES IN HUMAN BRAIN TISSUE HOMOGENATES. THE EVIDENCE OF PEROXIDE FORMATION

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EINLEITUNG

BESTIMMTE Beobachtungen der pathologischen Anatomie geben Anlass zum näheren Studium von Autoxydationsvorgängen an ungesättigten Bausteinen des menschlichen Gehirns und der mit diesen verbundenen chemisch-physikalischen Veränderungen.

Wie wir in früheren Untersuchungen gezeigt haben, erleiden Glycerinphosphatide des menschlichen Gehirns durch Aufnahme von Sauerstoff eine Viskositätserhöhung (SCHULZ und WILKE, 1955, 1956; WILKE und SCHULZ, 1955). Dieser Vorgang zeigt die typischen Merkmale einer Autoxydation, nämlich Beschleunigung durch radikalbildende Stoffe (z.B. Peroxyde) und Inhibierung durch Hydrochinon. Unter Molekülverknüpfung bilden sich dabei polymere, wahrscheinlich vernetzte Produkte, die eine Viskositätssteigerung verursachen. Für die Viskositätszunahme als Folge der Autoxydation wird nur ein sehr geringer Prozentsatz des normalerweise im Gehirn vorhandenen Sauerstoffs benötigt.

Die Gewebsatmung kann unter verschiedenen krankhaften Bedingungen gestört sein (KETY, 1948, 1950, 1955, 1956, und andere). In Fällen, bei welchen sich in tabula als wesentlichstes Merkmal der Hirngewebsveränderung eine charakteristische Konsistenzzunahme (NEUMANN, 1933) findet, liegt es nahe, Autoxydationsvorgänge in Betracht zu ziehen.

Als Beweis für eine Autoxydation kann der Nachweis der im Verlaufe der Autoxydation gebildeten Peroxyde gelten. Mit Hilfe geeigneter Methoden wurde die Bildung der Peroxyde als Folge der Autoxydation in verschiedenen Systemen verfolgt.

Unter *physiologischen* Verhältnissen ist zwar das Auftreten von Peroxyden im Gewebe noch nicht nachgewiesen. In fast allen Organen kommen jedoch Fermente vor (Katalasen, Peroxydasen), die Wasserstoffperoxyd und organische Hydroperoxyde zersetzen (THEORELL, 1948) und unter normalen Bedingungen deren Anreicherung verhindern. Z.B. ist die Katalase *in vitro* befähigt, *tert.*-Butylhydroperoxyd zu zersetzen (KERN, JERCHEL, SCHMIDT und EBERHARD, 1956).

Unter *pathologischen* Bedingungen ist die Peroxydation menschlichen Fettgewebes, z.B. bei peripheren Venenkrankheiten (JESSEN, GLAVIND, HARTMANN und DAM, 1951), beschrieben, ebenso das Vorkommen von Lipoperoxyden in atherosklerotischen

Aorten (GLAVIND, HARTMANN, CLEMMESSEN, JESSEN und DAM, 1952) und nach Einwirkung von ultraviolett-und Röntgenstrahlen, von Hitze, Kälte und Verbrennung und bei entzündlichen Prozessen in der Haut (DOBOULOZ und DUMAS, 1954).

Unter *experimentellen* Bedingungen wurde Peroxydbildung in Homogenaten von Leber, Niere und Gehirn beobachtet (KOHN und LIVERSEDGE, 1944; BERNHEIM, BERNHEIM und WILBUR, 1948; WOLFSON, WILBUR und BERNHEIM, 1956).

Die von den Autoren benutzten Nachweismethoden für Peroxyde erwiesen sich für unsere Zwecke nur bedingt anwendbar. Angesichts des Mangels direkter Nach-

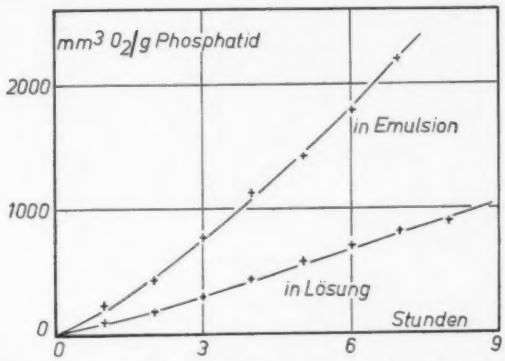

ABB. 1.—Autoxydation von Gehirnphosphatiden bei +36°C in Emulsion und in Lösung.

weismethoden im Hirngewebe war es ratsam, die Untersuchungen wie bisher schrittweise vorzunehmen. Die vorliegende Arbeit hatte das Ziel, unsere früheren Untersuchungen an Gehirnphosphatiden und daraus isolierten hochungesättigten Fettsäuren auf Gewebshomogenate zu übertragen. Wegen der Kompliziertheit der Systeme wurde zunächst noch das Verhalten *emulgierter* Gehirnphosphatide untersucht.

Die Autoxydation der Gehirnphosphatide in Emulsion

Die Autoxydation ungesättigter Verbindungen als solcher und die Effekte zugefügter Katalysatoren und Inhibitoren sind bekannt. Da diese Reaktion für die Zielsetzung unserer Untersuchung von grundsätzlicher Bedeutung ist, wurde sie speziell am Beispiel der menschlichen Gehirnphosphatide nochmals überprüft.

Unter bestimmten Bedingungen lassen sich Gehirnphosphatide ohne Zusatz fremder Emulgatoren in Wasser, physiologischer Kochsalzlösung oder Ringerlösung emulgieren. Diese Emulsionen sind sehr stabil und bei tiefer Temperatur wochenlang haltbar. (Herstellung siehe experimenteller Teil).

Die Autoxydationsversuche wurden in einer Warburgapparatur bei 36°C vorgenommen. Für jeden Versuch wurden 3 ml einer 5%-igen Emulsion eingesetzt (entsprechend 150 mg Phosphatid). Jeder Messpunkt ist der Mittelwert aus zwei bis vier unabhängigen Versuchen.

In Abb. 1 ist die Sauerstoffaufnahme pro Gramm Phosphatid in Abhängigkeit von der Zeit dargestellt. Zum Vergleich wurde die Sauerstoffaufnahme eingezeichnet, die am gleichen Phosphatidpräparat gemessen wurde, wenn dieses nach der früher angewandten Methode in Palmitinsäure-Methylester gelöst war. Die Abbildung zeigt, dass Gehirnphosphatide auch in Emulsion autoxydierbar sind und dass die Autoxydation unter diesen, den physiologischen Verhältnissen näher stehenden

Bedingungen sogar noch schneller verläuft als in homogener Lösung. Die Ansicht, dass Autoxydationsvorgänge auch *in vivo* prinzipiell möglich sind, wird durch diese Beobachtung gestützt.

Durch die beschleunigende, bzw. hemmende Wirkung von Zusätzen wird bewiesen, dass es sich bei dieser Sauerstoffaufnahme um eine Autoxydationsreaktion handelt. Als positive Katalysatoren (Beschleuniger) wirken auch hier Peroxyde, als negative Katalysatoren (Inhibitoren) z.B. Hydrochinon (siehe Tabelle 1). Auch Tocopherolacetat wirkt als Autoxydationsinhibitor. An Gehirnphosphatiden in Emulsion ergibt sich somit das gleiche Bild wie bei entsprechenden Versuchen mit hochungesät-

TABELLE 1.—DIE WIRKUNG VON ZUSÄTZEN AUF DIE AUTOXYDATION VON GEHIRNPHOSPHATIDEN IN EMULSION

(Jeweils 3 ml 5%-ige Emulsion; Temp.: 36°C)

Zusatz	Autoxydationszeit (Stunden)	Aufgenommener O_2 (mm^3/g Phosphatid)
Kein	8	2650
15 mg Benzoylperoxyd	8	8700
2·5 mg Wasserstoffperoxyd	8	9150
Kein	8	2650
15 mg Hydrochinon	8	950
20 mg Tocopherolacetat	8	2000
15 mg Benzoylperoxyd	4	3230
15 mg Hydrochinon, nach 4 Std. zugesetzt	7	3470
2·5 mg Wasserstoffperoxyd	4	6300
15·0 mg Hydrochinon, nach 4 Stunden zugesetzt	7	6650

tigten Fettsäuren und mit Gehirnphosphatiden in Lösung. Demnach dürfte in allen Fällen der gleiche Reaktionsmechanismus, nämlich ein radikalinduzierter autokatalytisch verlaufender Autoxydationsprozess, zugrunde liegen (SCHULZ und WILKE, 1956). Es ist in diesem Zusammenhang nicht so wesentlich, dass die Reaktionsgeschwindigkeiten in Lösung und in wässriger Emulsion verschieden sind. Vielmehr scheint uns die Feststellung wichtig, dass die Autoxydation der Phosphatide in wässriger Emulsion nach ähnlichen Reaktionsmechanismen abläuft.

Nachweis von Peroxyden in autoxydierten Phosphatidemulsionen

Wie bereits ausgeführt wurde, kann das Auftreten von Peroxyden für den Nachweis einer Autoxydationsreaktion herangezogen werden. Es wäre also erforderlich, die als Primärprodukte einer Autoxydation auftretenden Peroxyde in Phosphatidemulsionen, in Gewebshomogenaten und schliesslich im Hirngewebe selbst nachzuweisen und quantitativ zu bestimmen. Die analytische Bestimmung der Peroxyde könnte entscheiden, ob bei bestimmten krankhaften Veränderungen des Hirngewebes Autoxydationsvorgänge beteiligt sind. Die quantitative Peroxydbestimmung an krankhaft verändertem Gewebe würde auch Rückschlüsse zulassen über die Menge des dabei

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aufgenommenen Sauerstoffs. Die bisher von uns geprüften Methoden ermöglichen jedoch keine exakte quantitative Auswertung.

Zur *quantitativen* Bestimmung von Peroxyden sind eine ganze Reihe von chemischen Methoden bekannt. Die zur Bestimmung von Peroxyden in Fetten angewandten Methoden sind jedoch nicht ohne weiteres bei Phosphatiden brauchbar, insbesondere nicht bei Gehirnphosphatiden.

So erwiesen sich jodometrische Methoden wie etwa diejenige von LEA (1952) infolge ihrer geringen Empfindlichkeit von vornherein als unbrauchbar. Das von HARTMANN und GLAVIND (1949) u. GLAVIND und HARTMANN (1955) vorgeschlagene Leuco-2:6-dichlorphenolindophenol eignete sich für

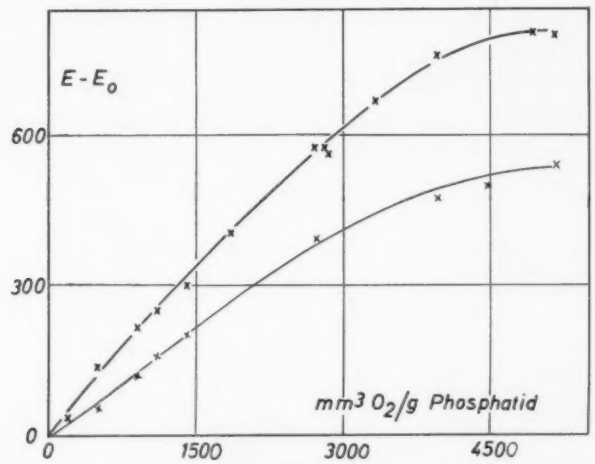

ABB. 2.—Peroxydgehalt autoxydierter Phosphatidemulsionen in Abhängigkeit von der Sauerstoffaufnahme bei der Autoxydation. *obere Kurve*: Thiocyanatmethode; *untere Kurve*: Thiobarbitursäure–Methode. $E - E_0$ = Zunahme der Extinkton bei 546 mμ (berechnet auf 1 g Phosphatid).

Untersuchungen an Eilecithin, versagt aber völlig bei Gehirnphosphatiden. Die von HILLS und THIEL (1946) u. HILLS und WILKINSON (1946) vorgeschlagene Bestimmung mit Ferrochlorid und Ammoniumthiocyanat ist ebenfalls auf Eilecithin anwendbar, wird aber von Gehirnphosphatiden stark gestört.

Versetzt man die Lösung eines Peroxyds (z.B. *tert.*-Butylhydroperoxyd) mit Eisen-(II)-chlorid und Ammoniumthiocyanat, so ist die Extinktion des gebildeten Eisen-(III)-thiocyanats proportional dem Peroxydgehalt (HILLS und THIEL, 1946; GLAVIND und HARTMANN, 1951). Durch die Anwesenheit von Eilecithin wird die Bestimmung nicht beeinflusst. Setzt man aber einer Lösung von *tert.*-Butylhydroperoxyd bekannten Gehaltes *Gehirn*phosphatide zu, dann wird die Extinktion herabgesetzt und der gefundene Peroxydgehalt weicht von der vorgegebenen Menge stark ab. Die Thiocyanatmethode wird also bei Peroxydbestimmungen in Gehirnphosphatiden gestört. Nur bei niedrigen Konzentrationen der Hirnphosphatide (etwa 1 mg/ml Messansatz) können noch Relativwerte für den Peroxydgehalt der Hirnphosphatide erhalten werden (Ausführung siehe experimenteller Teil).

Bei Einhaltung bestimmter Konzentrationsbedingungen lässt sich zeigen, dass mit steigender Sauerstoffaufnahme auch der Peroxydgehalt der Phosphatidemulsionen, gemessen an der Extinktion der Eisen-Thiocyanatlösung, ansteigt (siehe Abb. 2). Dieser Anstieg erfolgt nur im Anfangsstadium linear; bei höheren Sauerstoffaufnahmen besteht keine lineare Abhängigkeit mehr. Der mittels Thiobarbitursäure (TBA) ausgeführte Peroxydnachweis von BERNHEIM *et al.* (1948) und WOLFSON *et al.* (1956), konnte unverändert auch auf Gehirnphosphatide übertragen werden. Diese Reaktion liefert aber ebenfalls nur Relativwerte für den Peroxydgehalt (siehe Abb. 2).

Die Neigung und der lineare Bereich der in Abb. 2 wiedergegebenen Kurven hängen von der Isolierung und Aufarbeitung der Gehirnphosphatide ab und sind von Fall zu Fall verschieden.

Zum *qualitativen* Nachweis der Peroxyde in autoxydierten Phosphatidemulsionen benutzten wir ferner die Fähigkeit der Peroxyde, die Polymerisation bestimmter ungesättigter Verbindungen, z.B. Acrylamid, auszulösen. Die Bildung von polymerem Acrylamid äussert sich in einer Viskositätserhöhung der Lösung, die schliesslich zu einer Gallerte erstarrt. Bei Ausschluss von Sauerstoff oder Peroxyden tritt keine Polymerisation ein.

Die Versuche zeigten, dass bei Zusatz von autoxydierten Phosphatidemulsionen tatsächlich eine Gallertbildung eintritt (siehe experimenteller Teil).

Zusammenfassend kann festgestellt werden: die Thiocyanatmethode und die TBA-Methode ermöglichen zwar keine quantitative Bestimmung des gebundenen Sauerstoffs; es kann aber doch mit ihnen gezeigt werden, dass als Folge der Autoxydation emulgierter Gehirnphosphatide Peroxyde entstehen. Die Fähigkeit der autoxydierten Emulsionen, Polymerisationen auszulösen, ist als weiterer unabhängiger Beweis für die Bildung von Peroxyden zu betrachten.

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Autoxydation und Peroxydbildung in Hirngewebshomogenaten

Bei der Übertragung der an Phosphatidemulsionen angestellten Untersuchungen auf Homogenate möglichst frischer, unfixierter menschlicher Gehirne ist zunächst mit Atmungsvorgängen zu rechnen. Ferner kommen andere Einflüsse wie Alter, Grundkrankheit und schliesslich auch postmortale autolytische Vorgänge hinzu. Eine Abgrenzung dieser Einflüsse und eine sichere Auswertung ist erst durch Sammlung und Sichtung eines umfangreicheren Materials möglich. Hier soll über vorläufige Ergebnisse berichtet werden, die als gesichert gelten können.

Aus der Stirnregion unfixierter menschlicher Gehirne wurde Rinde und Mark getrennt in phosphatgepufferter Ringerlösung homogenisiert. Die Sauerstoffaufnahme wurde mit der 'direkten Methode' von Warburg gemessen (UMBREIT, BURRIS und STAUFFER, 1951).

Bei allen Versuchen ergab sich eine Sauerstoffaufnahme, die geringer ist als die für frische Hirngewebsschnitte in der Literatur angegebenen Werte (WARBURG, POSENER und NEGELEIN, 1924; HOLMES, 1930; DIXON und MEYER, 1936; HIMWICH, BAKER und FAZEKAS, 1939; OPITZ und SCHNEIDER, 1950; ELLIOT und SUTHERLAND, 1952). Diese Unterschiede führen wir darauf zurück, dass bei unseren Versuchen anstelle von Schnitten Homogenate eingesetzt wurden (QUASTEL und WHEATLEY, 1932; DIXON und MEYER, 1936; HIMWICH, BAKER und FAZEKAS, 1939; OPITZ und SCHNEIDER, 1950). Ferner stammten die Proben von Sektionsmaterial, das erst vierzehn bis vierundzwanzig Stunden nach dem Tode entnommen werden konnte.

Übereinstimmend mit den bekannten Atmungsgrössen von Hirnschnitten nehmen die Rindenhomogenate zehn-bis zwanzigmal soviel Sauerstoff auf wie die Markhomogenate (vergl. Tabelle 2).

Um zu prüfen, ob die gemessene Sauerstoffaufnahme lediglich einer Atmung zuzuschreiben ist, wurde in Parallelversuchen den Ansätzen Pentobarbital-Natrium (Nembutal, Fa. Abbott, London) zugefügt. Pentobarbital-Natrium wird in der Humanmedizin als Sedativum und in der Veterinärmedizin als Narkosemittel verwendet. Nach WILKINS, FEATHERSTONE, GRAY, SCHWIDDE und BROTMAN (1949)

TABELLE 2.—SAUERSTOFFAUFNAHME UND PEROXYDBILDUNG (TBA-WERTE) VON MENSCHLICHEN HIRNHOMOGENATEN

(Jeweils 3 ml Homogenat (*ca.* 80 mg Trockensubstanz) in Ringer-Phosphatlösung; pH 7·6 mit 0·2% Glucose. Warburggefässe; im Einsatz 0·2 ml 5%-ige KOH Schüttelgeschwindigkeit: 82/min; Temp.: +36°C; Gasfüllung: Sauerstoff. Differentialmanometer)

Sekt. Nr.	Homogenat aus	Zusatz: 0·1 ml Nembutal (6 mg Pentobarbital-Natrium)	Sauerstoffaufnahme (mm^3/g Trockensubstanz) nach				TBA-Werte ohne O_2-Behandl.	TBA-Werte nach 8 Stunden O_2-Behandl.
			1 Stunden	2 Stunden	4 Stunden	8 Stunden		
814	Rinde	ohne	3250	6500	11,100	16,900	13	110
		mit	560	750	1050	1850	13	110
	Mark	ohne	220	355	580	840	13	10
816	Rinde	ohne	3800	7300	13,000	18,000	23	190
		mit	300	600	1050	1550	23	150
	Mark	ohne	150	250	400	500	25	20
819	Rinde	ohne	3500	6450	13,550	16,950	20	230
		mit	400	750	1450	2100	20	210
	Mark	ohne	350	500	550	—	18	18
824	Rinde	ohne	5000	9500	17,650	24,150	23	260
		mit	500	750	1550	2200	23	200
	Mark	ohne	410	700	1090	1610	16·5	14
		mit	100	200	350	600	16·5	18·5
828	Rinde	ohne	4500	9500	18,200	26,300	27	170
		mit	500	750	1250	1800	27	130
	Mark	ohne	200	250	250	900	22	17
		mit	100	150	250	400	22	20

sollen 0·08 und 0·12% die Atmung vollständig hemmen. In unseren Versuchen wird durch 0·2% Pentobarbital-Natrium die Sauerstoffaufnahme der Rindenhomogenate zwar stark herabgesetzt aber nicht vollständig unterdrückt (Tabelle 2). Es wurde vermutet, dass der hierbei noch aufgenommene Sauerstoff nicht für Atmungsvorgänge sondern für Autoxydationsprozesse verbraucht wird. Diese Vermutung wird durch den Nachweis von Peroxyden mit Hilfe der TBA-Methode bestätigt. Mit ansteigender Sauerstoffaufnahme steigen die Peroxydgehalte der Homogenate an. Die Peroxydgehalte der 8 Stunden mit Sauerstoff behandelten Rindenhomogenate sind aber mit und ohne Zusatz von Pentobarbital-Natrium annähernd die gleichen (Tabelle 2). Wir ziehen hieraus den Schluss, dass in den *Rinden*homogenaten gleichzeitig mit der Atmung auch eine Autoxydation abläuft, welche sich nicht durch Pentobarbital-Natrium unterdrücken lässt und welche durch die Peroxydbildung nachweisbar ist.

Der Sauerstoffverbrauch der *Mark*homogenate wird durch den Zusatz von Pentobarbital-Natrium nur geringfügig beeinflusst. Da aber die TBA-Werte vor und nach der Sauerstoffbehandlung praktisch gleich sind, ist zu schliessen, dass in den

Markhomogenaten unter diesen Bedingungen keine nachweisbaren Autoxydationsreaktionen stattfinden.

Es kann noch auf andere unabhängige Weise gezeigt werden, dass mit der Sauerstoffaufnahme von Hirngewebshomogenaten eine Autoxydation verbunden ist.

Peroxyde haben bekanntlich die Fähigkeit, die Autoxydation ungesättigter Verbindungen zu beschleunigen (BOLLAND, 1948; KERN und WILLERSINN, 1954, 1955). Die Steigerung der Autoxydationsgeschwindigkeit ungesättigter Verbindungen nach Zusatz einer peroxydhaltigen Substanz stellt ein Mass für den Peroxydgehalt der Substanz dar.

Bei unseren Versuchen sind wir so verfahren, dass als autoxydierbare Verbindung eine Emulsion von Eilecithin (Merck) eingesetzt wurde. (Herstellung der Emulsion analog den im experimentellen Teil beschriebenen Phosphatidemulsionen). Diese Lecithinemulsion zeigte im Warburg-Versuch eine geringere Sauerstoffaufnahme als die Hirnphosphatide in Emulsion. Wurde den Ansätzen aber ein autoxydiertes Hirngewebshomogenat (Gesamthomogenat aus Rinde und Mark, 30 Stunden autoxydiert) zugesetzt, dann ergab sich eine beträchtliche Steigerung der Autoxydationsgeschwindigkeit. Die Sauerstoffaufnahme war höher als die Summe der von den einzelnen Komponenten aufgenommenen Menge Sauerstoff (Aufnahme Lecithin allein + Aufnahme Gesamthomogenat allein), die in Parallelversuchen bestimmt wurde. *Die erhöhte Autoxydationsgeschwindigkeit des Lecithins ist auf den Peroxydgehalt der autoxydierten Gewebshomogenate zurückzuführen.* Bei Einsatz verschieden stark autoxydierter Gewebshomogenate fanden wir eine entsprechend verschiedene Steigerung der Autoxydationsgeschwindigkeit des Lecithins.

Die Bildung von Peroxyden in menschlichen Hirngewebshomogenaten kann durch Zusatz von Tocopherol verhindert werden. Tocopherolacetat zeigt eine schwächere Wirkung als die nicht acetylierte Verbindung. Die durch Atmung bedingte Sauerstoffaufnahme wird durch Tocopherol nicht beeinflusst. Der Peroxydgehalt, gemessen an den TBA-Werten, kann unter der inhibierenden Wirkung von Tocopherol praktisch auf dem Ausgangswert bleiben (Tabelle 3).

In Rindenhomogenaten aus pathologisch veränderten menschlichen Gehirnen kann die Inhibierung der Autoxydation durch Tocopherol versagen (Tabelle 3).

Gewebshomogenate aus Gross- und Kleinhirn frisch getöteter Kaninchen zeigen ebenfalls mit fortschreitender Sauerstoffaufnahme einen Anstieg der Peroxydwerte. Die Hirngewebshomogenate wurden unmittelbar im Anschluss an die Tötung der Tiere hergestellt. Die erste Ablesung in der Warburg-Apparatur erfolgte eine Stunde nach dem Tode. Auch in frischen tierischen Hirngewebshomogenaten findet neben der Atmung eine Autoxydation statt, die schon in den ersten 30 Minuten einen Anstieg der TBA-Werte verursacht.

EXPERIMENTELLER TEIL

Die Chemikalien wurden analysenrein von der Fa. Merck, Darmstadt, bezogen.

Gewinnung der Hirnphosphatide. Glycerinphosphatide wurden nach der Methode von KLENK (1931–1954) aus menschlichen Gehirnen isoliert und unter Aceton in Stickstoffatmosphäre bei +3 – +5°C aufbewahrt.

Herstellung der Phosphatidemulsion. 2·5 g *in vacuo* von Aceton befreiten Phosphatids werden in einer grossen Porzellanreibschale, die durch mehrstündiges Einlegen zwischen Trockeneis stark abgekühlt worden war, in hartgefrorenem Zustand fein pulverisiert; unter ständigem Reiben wurden nach und nach 45 ml Ringerlösung zugesetzt. Die entstehende Mischung aus Eis und Phosphatid wird weiter kräftig durchgearbeitet, bis die Masse auftaut und dann in einen 50 ml-Messkolben

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TABELLE 3.—SAUERSTOFFAUFNAHME UND PEROXYDBILDUNG (TBA-WERTE) VON HOMOGENATEN AUS MENSCHLICHER HIRNRINDE

Jeweils 3 ml Homogenat (*ca.* 80 mg Trockensubstanz) in Ringer-Phosphatlösung pH 7·6 mit 0·2% Glucose. Warburggefässe: im Einsatz: 0·2 ml 5%-ige KOH; Schüttelgeschwindigkeit: 82/min. Temp.: +36°C; Gasfüllung: Sauerstoff; Differentialmanometer.)

Sektions Nr.	Zusatz	Sauerstoffaufnahme (mm^3/g Trockensubstanz) nach 1 Stunde	2 Stunden	4 Stunden	8 Stunden	TBA-Werte ohne O_2-Behandl.	nach 8 Stunden O_2-Behandl.	Alter (Jahre) Geschlecht	Pathologisch-anatomische Diagnose
838	ohne	3750	7450	12,400	20,650	25	260	54	Frische Unterbindung der A. mammaria bds. (Hypertonie). Hypertrophie und Dilatation des Herzens, Coronarsklerose. Alter Hinterwandinfarkt. Alter linsengrosser Erweichungsherd im N. lentiformis li., Sklerose der Hirnbasisgefässe.
	0·67% Tocopherol	3750	7650	12,700	18,800	25	20	♂	
	0·67% Tocopherol-acetat	3350	6550	11,600	19,250	25	190		
	0.2% Nembutal	450	900	1450	3750	25	150		
839*	ohne	2450	4550	7600	13,900	20	225	48	Magen-Carcinom, Lungen- und Knochenmetastasen, u.a. in der knöchernen Schädelbasis, Hirnschwellung.
	0·67% Tocopherol	2450	4550	7600	13,200	20	180	♀	
	1·33% Tocopherol	2450	4550	7600	13,700	20	165		
	0·2% Nembutal	300	500	1050	2000	20	165		
	1·33% Tocopherol + 0·2% Nembutal	300	500	1050	5250	20	40		
840	ohne	4700	9300	17,400	41,500	25	250	72	Schwere allgemeine Arteriosklerose. Frischer Myocardinfarkt. Ruptur der Vorderwand der li. Herzkammer. Hämatopericard. Sklerose der Hirnbasisgefässe.
	0·5% Tocopherol	5300	9850	17,950	44,500	25	17	♀	
	1·0% Tocopherol	4650	9500	17,500	33,800	25	15		
	0·2% Nembutal	400	750	1350	4550	25	170		
	1·0% Tocopherol + 0·2% Nembutal	400	750	1400	4350	25	20		
841	ohne	2100	4250	7450	22,100	15	105	81	Arteriosklerose. Thrombose der re. Vena femoralis. Lungenembolie.
	0·5% Tocopherol	1900	3850	7000	22,100	15	25	♀	
	0·2% Nembutal	350	450	800	5450	15	70		
	0·5% Tocopherol + 0·2% Nembutal	200	250	800	5350	15	30		
842*	ohne	1950	3800	6200	7450	20	190	61	Morbus Kimmelstiel-Wilson bei Diabetes mellitus mit Massenblutung (Apoplexie).
	0·08% Tocopherol	2050	3800	6150	7750	20	160	♂	
	0·5% Tocopherol	2100	3800	6000	7500	20	135		
	0·2% Nembutal	450	600	1050	1850	20	130		
	0·5% Tocopherol + 0·2% Nembutal	nicht gemessen				20	90		
843	ohne	4200	8600	15,500	22,000	15	195	59	Coronarsklerose, alter Vorderwand- und frischer Hinterwandinfarkt, geringgradiges Hirnödem.
	0·17% Tocopherol	4100	8200	16,000	21,900	15	35	♂	
	0·5% Tocopherol	4200	8350	16,200	22,600	15	25		
	0·2% Nembutal	300	700	1150	2050	15	135		
	0·5% Tocopherol + 0·2% Nembutal	200	450	900	1650	15	20		

* Keine Wirkung von Tocopherol, pathologisches Hirnmaterial.

gegeben, der vorher mit Stickstoff gefüllt wurde. Nach längerem Stehen im Eisschrank werden die an der Oberfläche der Emulsion angesammelten Gasblasen an der Wasserstrahlpumpe abgesaugt und das Volumen der Flüssigkeit auf 50 ml aufgefüllt, womit die Emulsion gebrauchsfertig ist.

Herstellung der Hirnhomogenate. Von dem frischen unfixierten Gehirn wurde nach Abtrennen der beiden Stirnpole die nachfolgende linke Frontalscheibe mit isotonischer NaCl-Lösung abgespült und Rinde und Mark säuberlich voneinander getrennt. Zum Homogenisieren wurden jeweils etwa 3 g Mark und etwa 6 g Rinde verwendet. Die gewogenen Stücke wurden mit Ringer-Phosphatlösung (pH 7·6) in einem Gewebemörser aus Glas bis zum Verschwinden der festen Bestandteile bearbeitet. Der entstehende Gewebsbrei wurde mit Ringerphosphat auf 30 ml aufgefüllt. Hiervon wurden für jeden Ansatz 3 ml verwendet. Zur Bestimmung des Trockengewichts der Homogenate wurden jeweils dreimal 3 ml im Trockenschrank bei +110°c bis zur Gewichtskonstanz getrocknet. Aus dem Gewicht des Rückstandes wurde unter Berücksichtigung des Trockengehaltes der Ringer-Phosphatlösung der Gehalt an Trockensubstanz das Gewebes berechnet. Die Auswertung der Messergebnisse wurde auf 1 g Trockensubstanz bezogen.

Herstellung von Ringer-Phosphatlösung pH 7·6. 8 ml einer Lösung von KCl (1·15%), 8 ml einer Lösung von $CaCl_2 \cdot 6\,H_2O$ (1·22%), 4 ml einer Lösung von KH_2PO_4 (2·11%) und 85 ml einer Lösung von $Na_2HPO_4 \cdot 2H_2O$ (1·78%) werden mit 0·9%-iger NaCl-Lösung auf 500 ml aufgefüllt, die Lösung aufgekocht, heiss filtriert und verschlossen.

Manometrische Messungen. Die Messung der Sauerstoffaufnahme erfolgte in einer Warburg-Apparatur bei +36°c in reinem Sauerstoff mit einer Schüttelfrequenz von 82/min. Für die Messung mit Phosphatidemulsionen wurden gewöhnliche Kastengefässe, für die Gewebshomogenate Gefässe mit Einsatz verwendet. Die Einsätze enthielten 0·2 ml 5%-ige Kalilauge. Das eingesetzte Volumen der Emulsionen oder Homogenate war immer 3 ml. Die Ansätze wurden 25 Minuten bei Zimmertemperatur mit Sauerstoff durchströmt, 5 Minuten zum Temperaturausgleich bei +36°c geschüttelt und dann die Manometer eingestellt.

Peroxydbestimmungen mit der Thiocyanatmethode (modifiziert nach HILLS und Mitarbeiter, 1946). Die einzusetzende Phosphatidlösung gewinnt man durch Auflösen von 50 mg trockenen Phosphatids oder durch Vermischen von 1 ml einer 5%-igen Emulsion (s.o.) mit Alkohol/Chloroform (3 : 5 v/v) und Auffüllen auf 25 ml. Aus autoxydierten Emulsionen wird vor Herstellen der Lösung der Sauerstoff durch Einblasen von Stickstoff vertrieben.

Von der so entstandenen 0·2%-igen Lösung werden 5 ml, entsprechend 10 mg Phosphatid, zur Messung eingesetzt. Dazu werden nacheinander gegeben:

2·5 ml Alkohol/Chloroform (3 : 5 v/v)

2·0 ml 30%-iges Ammoniumthiocyanat in Methanol

0·5 ml ca. 0·2%-ige Lösung von $FeCl_2$ in *ca.* 10%-igem Eisessig

Alle Lösungen werden vor Gebrauch mit Stickstoff entlüftet

3–5 Minuten nach Zufügen der $FeCl_2$-Lösung und Durchmischen wird die Extinktion bei 546 mμ und 1 cm Schichtdicke gemessen. Vergleichslösung: in obiger Mischung statt der Phosphatidlösung 5·0 ml Alkohol/Chloroform.

Herstellung der $FeCl_2$*-Lösung.* 700 mg Mohrsches Salz $Fe(SO_4)_2\,(NH_4)_2 \cdot 6\,H_2O$ werden in 40 ml redest. Wasser (sauerstoff-frei) gelöst und 10 ml Eisessig zugesetzt. Dann werden (zunächst tropfenweise, dann rascher, unter schnellem Umschwenken) 880 mg $BaCl_2 \cdot 2\,H_2O$ in 25 ml Wasser zugesetzt und das Ganze durch Einleiten von N_2 entlüftet. Das ausgefallene $BaSO_4$ wird abzentrifugiert; die verbleibende Lösung von $FeCl_2$ ist nach nochmaligem Entlüften gebrauchsfertig und mehrere Tage haltbar. Falls die Extinktion der oben beschriebenen Vergleichslösung gegen Alkohol/Chloroform mehr als 0·1 beträgt, sollte eine neue Lösung angesetzt werden.

Auswertung. Die bei 546 mμ gemessene Extinktion wird durch die im Messansatz herrschende Konzentration (in unseren Versuchen immer 10^{-3} g/ml) dividiert und ergibt den Wert E. Da auch das nicht behandelte Phosphatid bei obiger Messmethode einen Extinktionswert E_0 ergibt, wird dieser von E abgezogen. $E - E_0$ stellt dann ein Mass für die bei der Autoxydation entstandene Menge Peroxyd dar.

Peroxydbestimmungen mit der Thiobarbitursäure-Methode (nach WOLFSON, WILBUR und BERNHEIM, 1956). In einem 10–15 ml fassenden Zentrifugenglas werden 1 ml Phosphatidemulsion, bzw. Gewebshomogenat, 2 ml 20%-ige Trichloressigsäure und 4 ml 0·66%-ige 2-Thiobarbitursäure (Fa. Th. Schuchardt, München) (unter Erwärmen in Wasser gelöst) 15 Minuten in ein kochendes Wasserbad gestellt (Reagenzglasgestell aus Blech, Siedesteinchen einwerfen). Danach wird die entstandene

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Fällung abzentrifugiert, die überstehende Lösung mit *aqua dest.* auf 10 ml aufgefüllt und bei 546 mμ gegen Wasser als Vergleichslösung kolorimetriert. Als TBA-Wert wird die auf 1 g/ml bezogene Extinktion (bei 1 cm Schichtdicke) bezeichnet.

Die Genauigkeit der Methode wird bei Gewebsuntersuchungen dadurch beeinträchtigt, dass ein Teil des gebildeten Farbstoffs von den ausgefällten Eiweissverbindungen adsorbiert wird. Die Farbreaktion erfolgt mit autoxydierten Gehirnphosphatiden, Eilecithin und Hirngewebshomogenaten—aber nicht mit niederen Peroxyden, wie Benzoylperoxyd (GLAVIND und HARTMANN, 1951).

Für die Extinktionsmessungen wurde das lichtelektrische Photometer 'Eppendorf' mit Glasküvetten von 1 cm Schichtdicke verwendet.

Peroxydnachweis durch Polymerisationsauslösung. 3·0 ml einer im Warburg-Apparat autoxydierten Phosphatidemulsion werden mit 300 mg Acrylamid versetzt und die Luft durch Stickstoff verdrängt. Nach 2 Stunden wird die Emulsion bei Zimmertemperatur viskos und erstarrt nach 4 Stunden zu einer Gallerte. Lösungen von Acrylamid in nicht autoxydierten Phosphatidemulsionen zeigten erst nach 9 Stunden eine Viskositätserhöhung. Vergleichsversuche mit Acrylamid in Ringerlösung waren unter gleichen Bedingungen nach 48 Stunden unverändert.

ZUSAMMENFASSUNG DER ERGEBNISSE UND DISKUSSION

Gehirnphosphatide sind sowohl in homogener Lösung als auch in Emulsion autoxydabel. Die Autoxydation wird durch die Bildung von Peroxyden bewiesen. In autoxydierten Phosphatidemulsionen lassen sich die primär gebildeten Peroxyde mit Hilfe der Thiocyanat-Methode, der Thiobarbitursäure-Methode und durch ihre polymerisationsauslösende Wirkung auf z.B. Acrylamid qualitativ nachweisen. Die quantitative Bestimmung stösst in den untersuchten Systemen auf grosse Schwierigkeiten. Vermutlich wird mit fortschreitender Sauerstoffaufnahme ein Teil der gebildeten Peroxyde u.a. durch Bildung polymerer Peroxyde weiter verändert und dadurch der quantitativen Bestimmung entzogen.

Gewebshomogenate aus menschlichen Gehirnen nehmen ebenfalls Sauerstoff auf. Träger der Sauerstoffaufnahme ist hier überwiegend die Hirnrinde. Der aufgenommene Sauerstoff wird nicht nur zur Atmung verbraucht, sondern gleichzeitig zur Autoxydation. Der Anteil der Sauerstoffaufnahme, der auf die Atmung zurückzuführen ist, kann durch Pentobarbital vollständig gehemmt werden. Der nicht hemmbare Anteil beruht auf Autoxydation. Diese wird bewiesen durch die Bildung von Peroxyden, welche mit der TBA-Methode und durch die Beschleunigung der Lecithinautoxydation nachweisbar sind. Die Bildung von Peroxyden kann durch Tocopherol verhindert werden; in pathologisch verändertem Hirnmaterial kann die inhibierende Wirkung des Tocopherols versagen.

Die Feststellung, dass die durch Autoxydation bedingte Sauerstoffaufnahme und damit verbunden die Peroxydbildung überwiegend in der grauen Substanz stattfindet—steht in guter Übereinstimmung mit der von anderen Autoren gefundenen topographischen Verteilung hochungesättigter Bausteine im Gehirn (ALSTERBERG, 1948; BLANCHER, LE BRETON und MAY, 1952; BLANCHER und DE VAUX-SAINT-CYR, 1953; GENSEL, 1958).

Mangels zuverlässiger histochemischer Methoden ist es im Augenblick noch nicht möglich, den direkten Nachweis der Autoxydation, etwa über die Peroxydbildung, im Gewebs*schnitt* zu führen. Die Untersuchungen an Phosphatidemulsionen und Gewebshomogenaten zeigen die methodischen Schwierigkeiten des Peroxydnachweises selbst unter Autoxydationsbedingungen, die man experimentell in der Hand hat und sogar katalytisch steuern kann.

Die *quantitative* Deutung der Versuche an Hirngewebshomogenaten sowie die

Zuordnung zu bestimmten Altersklassen und krankhaften Gewebsveränderungen erfordert ein umfangreicheres Untersuchungsmaterial als es bis jetzt vorliegt.

Das Studium von Autoxydationsvorgängen ungesättigter Bausteine des menschlichen Gehirns und der damit verbundenen chemisch-physikalischen Veränderungen dürfte die Möglichkeit bieten, die Frage irreversibler Gewebsveränderungen bei Störungen des Gewebsstoffwechsels von der 'Substrat'-Seite, d.h. von morphologischer Seite, neu zu beleuchten und bestimmte bisher ungeklärte Beobachtungen der pathologischen Physiologie und pathologischen Anatomie zu vereinen.

ZUSAMMENFASSUNG

Die Autoxydation von Gehirnphosphatiden ist sowohl in homogener Lösung als auch in Emulsion möglich. Die Sauerstoffaufnahme von Gewebshomogenaten aus menschlichen Gehirnen ist bedingt durch gleichzeitig ablaufende Atmungs- und Autoxydationsvorgänge. Die bei der Autoxydation von Phosphatidemulsionen und von Hirngewebshomogenaten gebildeten Peroxyde lassen sich mit verschiedenen Methoden nachweisen. In Übereinstimmung mit der topographischen Verteilung hochungesättigter Bausteine im Gehirn finden Autoxydation und Peroxydbildung vorwiegend in der grauen Substanz statt.

SUMMARY

The autoxidation of brain phosphatides proceeds in homogeneous solution as well as in emulsions. The uptake of oxygen by tissue homogenates of human brains is due to simultaneous respiration and autoxidation. The peroxides formed during the autoxidation of phosphatide emulsions and brain tissue homogenates can be demonstrated by different methods. In agreement with the topographical distribution of highly unsaturated compounds in the brain, autoxidation and peroxide formation occur particularly in the grey matter.

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THE LIBERATION OF ACTIVE ENZYMES FROM BRAIN TISSUE BY LYSOLECITHIN

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IN 1951, ORD and THOMPSON described the ability of a number of surface-active agents to 'clear' the opaque supernatants obtained by the centrifugation of water homogenates of whole rat brain. In an attempt to purify the true cholinesterase of brain they further showed that a non-ionic surface-active agent, acetyl alcohol-polyoxyethylene condensate, was able to 'solubilize' this enzyme without causing any inactivation.

GROSSE and TAUBÖCK (1942) had earlier briefly reported that a brain *brei* suspended in physiological saline can be cleared with lysolecithin. A more detailed description of this action of lysolecithin on nervous tissue has recently been given by WEBSTER (1957), who has shown that when uncentrifuged homogenates of whole brain tissue are incubated at 38°C with lysolecithin, a very rapid and almost complete 'clearing' of the opaque homogenate occurs; following a 2 hr incubation with 0·007 M-lysolecithin, the optical transmission, using a neutral grey filter, of a 1 : 20 homogenate of rat brain in 0·025 M-sodium bicarbonate is equal to about 80 per cent of the optical transmission of water, while about 60 per cent optical transmission is reached after only 10 min incubation; the optical transmission of the homogenate at zero time was 5 per cent of that of water.

In view of this 'solubilizing' effect on the greater part of the components of the neurones, glial cells and myelin sheath lipids present in these homogenates, it seemed of interest to determine next what effects, if any, the lysolecithin, and the lysis produced by this compound, might have on the enzymic activities of the original homogenates. WEIL (1930) had earlier studied the effect of cobra venom, now known to contain a phospholipase *A*, on segments of spinal cord, and MORRISON and ZAMECNIK (1950), on the basis of similar observations, had suggested that if lysolecithin were to be formed inside the nervous system, localized demyelination might be expected to occur. We have therefore, in the first instance, been primarily interested in those enzymes which, if released into solution, might be expected to exert a further destructive action on cells or cell membranes, or which might interfere with the normal processes of excitation and transmission in unaffected nervous tissue. We have begun by assaying in lysolecithin-cleared preparations the levels of activity of the cholinesterases and of mono-amine oxidase, enzymes which are or may be concerned with the inactivation of transmitter or excitor substances, and of the proteinases, esterases, phosphatases and transaminases, which might be expected, if working in an unco-ordinated fashion, to exert degradative changes on the normal cells. A preliminary account of part of this work has already been published (MARPLES, THOMPSON and WEBSTER, 1957). From the point of view of any possible pathological significance, it would clearly be desirable to know what effect, if any, lysolecithin

might have on more intact brain preparations. For this reason we have also studied the effect of this compound on brain slices in order to determine whether it enhances the release of active enzymes from the slices into the suspending fluid.

As it has recently been shown (MARPLES and THOMPSON, 1958) that purified lysolecithin, prepared from ovolecithin by the method of LONG and PENNY (1957), may contain significant phospholipase *A* activity (see also HANAHAN, RODBELL and TURNER, 1954) we have in most of the present experiments, used re-crystallized lysolecithin which is free from detectable phospholipase *A* activity; mention is made below whenever the uncrystallized compound was used.

EXPERIMENTAL

Preparation of brain homogenates

Homogenates of whole rat brain (1 g of tissue in 3 ml of 0·025 M-$NaHCO_3$) were prepared with a modified Potter–Elvehjem type of homogenizer. The homogenate was divided into two parts, to one of which was added an emulsion of lysolecithin in 0·025 M-$NaHCO_3$, and to the other only the $NaHCO_3$ solution, so that in each case the dilution of the brain in the final mixture was 1 in 5. The final concentration of lysolecithin in the mixture was 87 mg/g of brain.

These brain preparations were then incubated at 38° for 60 min. At the end of this time the tubes containing the lysolecithin showed maximal clearing. Samples from each tube were taken for the various enzyme estimations.

Brain slice experiments

Slices of rat cerebrum were cut free-hand with a safety-razor blade moistened with the buffer solution in which they were subsequently suspended. The slices were transferred from the blade to a vessel containing 10–15 ml of the buffer. After draining excess fluid from them they were then transferred to tared Warburg flasks containing 2·7 ml of the bicarbonate buffer, pH 7·4 (containing 0·011 M-glucose), described by DICKENS and GREVILLE (1935), and the flasks re-weighed (weights of slices added varied from 50–150 mg). The vessels were then gassed with either 95% N_2 + 5% CO_2 or 95% O_2 + 5% CO_2 mixtures. After temperature equilibration (38°) the lysolecithin was tipped in from the side-bulbs, and the slices exposed to its action for 30 min, after which the contents of the flasks were pipetted into tubes and centrifuged at 3000 rev/min (1800 g) for 2 min. The clear supernatant solutions were then poured off for assay of true and pseudo-cholinesterase.

In all experiments duplicate flasks were set up for each concentration of lysolecithin, and duplicate assays were also carried out on the fluid in which slices had been incubated in the absence of lysolecithin. A homogenate of fresh slices in the buffer mixture was also prepared in order to determine the total cholinesterase activity of the preparation.

Estimation of enzyme activities

True and pseudo-cholinesterase. These were estimated manometrically at 38° and at pH 7·4 on 0·5 ml samples of the homogenates, or, in the case of the slice experiments, 2·0 ml of the centrifuged supernatant, using the Warburg technique. Acetyl-β-methylcholine chloride (0·03 M) (Savory & Moore Ltd.) and butyrylcholine perchlorate (0·03 M), prepared in this laboratory, were used as substrates for the true and pseudo-cholinesterases respectively. Activity is expressed as μl CO_2/g of tissue (wet wt.)/hr.

Tributyrinase. This also was measured on 0·5 ml samples, using the Warburg technique; the tributyrin (BDH) was placed in the side-arm of the Warburg flasks (0·03 ml/flask), and was covered with 0·17 ml of 0·025 M-$NaHCO_3$. Activity is expressed as μl CO_2/g per hr.

Mono-amine oxidase. This was measured manometrically on 1 ml samples of the homogenates, using tyramine hydrochloride (0·01 M) (L. Light & Co.) as substrate; in some of the experiments 0·2 ml KCN solution was added to the main chamber of the Warburg vessels to give a final concentration of 10^{-3} M, and 0·2 ml of a mixture of equal volumes of 2 M-KCN and 0·002 M-KOH was placed in the centre well. Activity is expressed as μl O_2/g per hr.

Acid and alkaline phosphatase. For these estimations the control and cleared homogenates were dialysed for 20 hr at 0–4°C against 200 vol. distilled water and 0·5 ml of the dialysed homogenate

was used for assay. Sodium phenyl phosphate (BDH) in a final concentration of 10^{-2} M was used as substrate; 0·1 M-carbonate–bicarbonate buffer (pH 8·9) was used for the alkaline phosphatase, and 0·1 M-acetate buffer (pH 5·5) for the acid phosphatase. The inorganic phosphate formed was estimated by the method of ERNSTER, ZETTERSTRØM and LINDBERG (1950) according to the procedure outlined by STRICKLAND, THOMPSON and WEBSTER (1956). Activity is expressed as μmoles inorganic P formed/g per hr.

Glycerylphosphorylcholine diesterase was also assayed on 0·5 ml samples of homogenates dialysed as described above. The glycerylphosphorylcholine was prepared in this laboratory as the crystalline $CdCl_2$ complex according to the method of TATTRIE and MCARTHUR (1955). The Cd was removed before use on an ion-exchange resin (Zeo-Karb 225, Na form). Enzyme activity was estimated as described by WEBSTER, MARPLES and THOMPSON (1957). Activity is expressed as μmoles choline liberated/g per hr.

TABLE 1.—ENZYME ACTIVITIES IN RAT BRAIN HOMOGENATES CLEARED WITH LYSOLECITHIN
(87 mg lysolecithin/g wet wt. of brain)

Enzyme	No. of experiments	Mean activity in Control homogenate	Mean activity in Cleared homogenate	Change in the cleared homogenate (%)
True cholinesterase (as μl CO_2/g per hr)	3	5697	6067	+6
Pseudo-cholinesterase (as μl CO_2/g per hr)	3	1438	1617	+12
Tributyrinase (as μl CO_2/g per hr)	3	6700	4077	−39
Monoamine oxidase (as μl O_2/g per hr)	3	−801	−429	−46
	2†	−312†	−179†	−43†
Alkaline phosphatase (as μmoles P/g per hr)	2	32·2	36·1	+12
	2*	39·0	42·1	+8
Acid phosphatase (as μmoles P/g per hr)	3	71·6	48·7	−32
	3*	87·1	49·7	−43
Glycerylphosphorylcholine diesterase* (as μmoles choline/g per hr)	3	18·3	19·5	+6
Proteinase* (as μmoles tyrosine equivalents/g per hr)	3	39·9	38·5	−3
Transaminase* (as μmoles aspartate produced/g per hr)	3	1248	1266	+1

* Brain homogenate cleared with uncrystallized lysolecithin in these experiments.
† In presence of 10^{-3} M-KCN.

Proteinase (cathepsin). Activity was determined on 0·2 ml samples of undialysed homogenates, using the method of ANSELL and RICHTER (1954). The results are expressed as μmoles of tyrosine equivalents liberated/g per hr.

Transaminase. Activity was determined on 0·5 ml samples of 1 in 30 dilutions of the homogenates, using the glutamic acid–oxaloacetic acid system and the chromatographic ninhydrin method described by KARMEN, WROBLEWSKI and LA DUE (1955). The results are expressed as μmoles aspartate formed/g per hr.

Reagents

Lysolecithin was prepared from ovolecithin; the latter was obtained from fresh eggs and was purified by chromatography on an alumina column according to the method of RHODES and LEA (1957). Conversion to lysolecithin was brought about by the action of the dried venom of the Cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*) on a 1 per cent solution of the purified lecithin in ether, using the method of LONG and PENNY (1957).

Crystallization of the lysolecithin was carried out from hot ethanol as described by SAUNDERS and THOMAS (1958).

RESULTS

The results of the enzyme estimations on different homogenates are shown in Table 1. It will be seen that in each experiment the activity of both the true and pseudo-cholinesterase is slightly higher in the cleared preparations than in the untreated control homogenate. Tributyrinase activity, on the other hand, shows a mean reduction of about 40 per cent in the 'cleared' as compared with the control specimens.

With mono-amine oxidase it is seen that the O_2 uptake in the presence of tyramine is reduced by about 40 per cent in the cleared preparations either in the presence or in the absence of CN^-. Alkaline phosphatase activity shows a slight activation, while acid phosphatase activity shows a reduction of about 33 per cent. The glyceryl-phosphorylcholine diesterase appears to be largely unaffected by the lysolecithin treatment. Proteinase and transaminase activities are also as great in the cleared as in the control preparations.

As MORRISON and ZAMECNIK (1950) had based their suggestions as to the possible consequences of the presence of lysolecithin in the central nervous system on experiments involving the incubation of slices of brain or spinal cord with cobra venom phospholipase *A*, it was decided to determine next whether incubation of slices of brain with lysolecithin also results in the liberation of any enzymes into the suspending solution. Experiments were, therefore, carried out with slices of rat cerebrum as described earlier. Table 2 summarizes the results of these experiments. It will be seen that a concentration of $1{\cdot}2 \times 10^{-3}$ M-lysolecithin causes a significant liberation of both the true and pseudo-cholinesterase into the suspending fluid, and that the amount liberated shows a somewhat variable increase as the concentration of lysolecithin is increased. In the absence of lysolecithin, incubation of the brain slices for 30 min under these conditions results in the liberation of only a small proportion of the total enzyme, 3 per cent and 6 per cent respectively, of the total true and pseudo-cholinesterase present. Essentially the same amount of enzyme is released whether the slices are incubated anaerobically or in the presence of oxygen.

Inspection at the end of the 30 min incubation showed an obvious reduction in the mass of the slices that had been incubated with 6×10^{-3} M-lysolecithin, while in the case of those that had been incubated with the highest concentration only a small fraction of the slices remained. Although no difference was apparent on naked-eye

TABLE 2.—LIBERATION OF CHOLINESTERASES FROM RAT BRAIN SLICES BY INCUBATION AT 38° FOR 30 MIN WITH LYSOLECITHIN

Enzyme	Activity of homogenate (μl/g per hr)	Percentage of activity of homogenate released into solution from slices by various concentrations of lysolecithin 0	1·2	6	18 $\times 10^{-3}$ M-lysolecithin
True cholinesterase	8838	5	17	—	53
	9160	4	23	45	52
	11,220	2	15	21	44
	9800	2	19	37	59*
	Mean	3	18	34	52
Pseudo-cholinesterase	1495	7	22	—	—
	1674	3	13	38	46
	1446	9	24	56	67
	1310	10	28	56	85*
	1193	3	17	41	59*
	Mean	6	21	48	64

* Brain slices treated with uncrystallized lysolecithin in these experiments.

inspection of the slices incubated with $1{\cdot}2 \times 10^{-3}$ M-lysolecithin, the addition of trichloroacetic acid to the centrifuged supernatant fluid caused the formation of a copious precipitate.

As a first step towards elucidating the mechanism of the inhibitory action of lysolecithin on certain enzymes, the degree of inhibition of tributyrinase produced by incubation with lysolecithin for varying periods of time was determined. It was found (Table 3) that the inhibition produced by 2·9 mg lysolecithin/ml was slowly progressive over 30 min.

On the other hand, the results given in Table 3 show that 32 μg moccasin venom/ml

TABLE 3.—INHIBITION OF TRIBUTYRINASE AFTER VARYING TIMES OF EXPOSURE TO LYSOLECITHIN AND MOCCASIN VENOM

(100 mg rat brain, homogenized in 2·7 ml 0·025 M-$NaHCO_3$, were warmed to 38° in Warburg flasks. Crystalline lysolecithin (8·7 mg) or moccasin venom (0·096 mg), together with 0·03 ml tributyrin, were then tipped in from side-bulbs. CO_2 evolutions were measured at times stated below. Results are expressed as inhibition per cent of tributyrinase activity in the control homogenate).

Inhibitor	Final conc. of inhibitor	Percentage inhibition at time (min) 5	10	15	20	30	50	60
Lysolecithin	2·9 mg/ml	5	9	9	17	22	21	—
		16	14	19	—	24	—	20
Moccasin venom	32 μg/ml	54	62	65	63	67	69	—
		52	64	67	—	68	—	70
		49	56	58	—	60	—	—

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produces a rapid inhibition of this enzyme. This concentration of venom, when acting on brain tissue under these conditions, yields less than 0·3 mg lysolecithin/ml, as judged by CO_2 evolution, in the first 5 min, so that the rapid inhibition of tributyrinase by the venom is unlikely to be due to lysophosphatides that might be formed by it from the phospholipids present in the brain homogenate: it would seem therefore to be due to a direct action of the venom on the enzyme.

Since it is known that phospholipase *A* activity is inhibited by ethylenediaminetetra-acetic acid (EDTA) (LONG and PENNY, 1957), the effect of this compound on the venom inhibition of tributyrinase was next studied; 10^{-2} M-EDTA was found to abolish completely the inhibitory action.

DISCUSSION

The mechanism of the clearing action of lysolecithin is still under investigation, but from what is known of its action on red blood cells and on mitochondria (COLLIER, 1952; NYGAARD, DIANZANI and BAHR, 1954), it would seem likely that one effect of this compound on brain tissue is to cause lysis of cell and mitochondrial membranes, with, possibly, the consequent release of particulate enzymes in soluble form. Although the activity of some of the enzymes we have studied is much reduced following clearing, a number of others appear to retain their full activity. This latter finding is in agreement with earlier observations made by BRAGANCA and QUASTEL (1953) on the inhibitory action of certain snake venoms on various enzyme systems. These workers set out to determine whether the neurotoxic action of cobra venom might result from a disturbance of enzymic activities in the nervous system. It is known that cobra venom contains a phospholipase *A* together with a number of other enzymes. In order to simplify the interpretation of the effects observed, BRAGANCA and QUASTEL heated the crude venom at 100° for 15 min, a procedure that fully inactivates all the enzymes known to occur in cobra venom other than the phospholipase *A*, which retains most of its activity after heating under these conditions. They found that heated venom inhibits the rate of O_2 uptake by brain homogenates in the presence of glucose, pyruvate, succinate, fructose, α-ketoglutarate and glutamate. Of the separate enzymes studied, succinate dehydrogenase, cytochrome oxidase and choline oxidase were found to be inhibited, although no reduction was found in hexokinase or glucose oxidase activity. They concluded that, in general, those enzymes whose activity is intimately associated with cell particles (mitochondria or microsomes) are inhibited by the heated venom, while the 'soluble' enzymes, whose activity is independent of cell structure, are unaffected. In view of the extensive work carried out by BRAGANCA and QUASTEL on the respiratory enzymes of the nervous system, we have not studied enzymes of this type. It is of interest to note however that in accordance with their conclusions about the dependence of venom-sensitive enzymes on cell structure, mono-amine oxidase, which we have found to be inhibited, is known to be largely located in the mitochondria (HAWKINS, 1952; COTZIAS and DOLE, 1951), while tributyrinase, also inhibited, is thought to be chiefly associated with the microsomes (OMACHI, BARNUM and GLICK, 1948). A large proportion of the acid phosphatases in rat liver homogenates is also linked with cytoplasmic particles, although, in this tissue at any rate, it can be released in an active, soluble form by ageing of the preparations or by repeated freezing and thawing (PALLADE, 1951; BERTHET and DE DUVE, 1951).

With each of the enzymes which show a reduction of activity in the cleared homogenate, crystallized lysolecithin, free from measurable phospholipase *A* activity, has been used for clearing. Results obtained with the alkaline and acid phosphatases and also with true and pseudo-cholinesterase (Tables 1 and 2, and other experiments not included) show however, that essentially the same results are obtained, with either crystallized or uncrystallized lysolecithin.

The effect of the small amount of phospholipase *A* present in the uncrystallized lysolecithin was further tested by determining the degree of clearing of brain homogenates produced by the addition of equal amounts (0·007 mole) of pure and crude lysolecithin; under the conditions described by WEBSTER (1957), incubation for 30 min with the pure lysolecithin resulted in a clearing of the homogenates to the extent that their optical transmission was equal to 79·5 per cent of that of water, while the crude compound gave a clearing equivalent to 85·5 per cent of the optical transmission of water, i.e. an increase of only 6 per cent. Incubation of homogenates with 195 μg of venom (calculated to equal the amount present in 13 mg of the crude lysolecithin, assuming complete co-precipitation of the venom) resulted in an increase in optical transmission of the homogenate of only 7 per cent; measurement of the CO_2 evolved during the clearing indicated the production of 0·0011 M-lysolecithin in the solution, i.e. a concentration which will itself cause clearing to about the observed extent. We have concluded therefore that the slightly greater clearing produced by the crude lysolecithin could be accounted for by the small extra amount of lysolecithin formed during the incubation by the phospholipase *A*. For all these reasons we have therefore not repeated the diesterase, proteinase and transaminase experiments with the pure lysolecithin.

Our results have shown that, in the case of tributyrinase, inhibition is progressive over 30 min. The rapid inhibition produced by moccasin venom, and the abolition of this inhibitory effect by EDTA, suggests the possibility that phospholipase *A* can itself inactivate this enzyme. In this connexion it will be recalled that KIELLEY and MEYERHOF (1950) had earlier shown that partially purified adenosinetriphosphatase preparations can be inhibited by the phospholipase *C* of *Cl. Welchii* toxin, and had concluded that a choline-containing phospholipid component present in these preparations was essential for their enzymic activity. Further work is in progress to extend these observations on the nature of the action of phospholipase *A* and of lysolecithin on tributyrinase.

The experiments with brain slices show that there is a significant liberation of both true and pseudo-cholinesterase activity from the tissue into the surrounding medium by incubation of the slices for 30 min with concentrations of lysolecithin (e.g. $1{\cdot}2 \times 10^{-3}$ M) which produce only a negligible increase in optical transmission of brain homogenates (WEBSTER, 1957). This release of enzyme activity also occurs rapidly, the greater part of the amount released by a given concentration of lysolecithin being liberated within 15 min after the addition of the lysolecithin to the slices. This, together with the observations of GREIG and GIBBONS (1956) mentioned below, would seem to indicate that an initial action of the lysolecithin on the cell membranes is to release these enzymes. Although some of the evidence concerning the intracellular localization of the true cholinesterase in the nervous system is conflicting, POPE and HESS (1957) have concluded that most of it is consistent with the view that this enzyme is located at the surface of the cell bodies and processes.

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A similar release of true cholinesterase from human erythrocytes has been reported by GREIG and GIBBONS (1956); these workers observed a 44 per cent decrease in cholinesterase activity when washed erythrocytes were incubated with $0{\cdot}9 \times 10^{-4}$ M-lysolecithin for 60 min. This is, admittedly, a much lower concentration of lysolecithin than we have found necessary for release from brain slices, but red cells are much more sensitive to the lytic action of lysolecithin than are nerve or muscle cells (TOBIAS, 1955; THOMPSON, unpublished observations).

The observations reported above would appear to support the prediction of MORRISON and ZAMECNIK (1950) that demyelination might be expected to occur if lysolecithin were to gain access to the nervous system. Cell lysis and solubilization of myelin certainly appear to occur, and they are also accompanied by the release of active enzymes which might be expected to extend the structural or functional damage. Concerning the production of lysolecithin within the nervous system, we have been unable to find any report in the literature of the presence of a phospholipase *A* in nervous tissue. The presence of such an enzyme in the liver and in the pancreas has, of course, been known for many years (FRANCOLI, 1934; GRONCHI, 1936; HANAHAN, 1952; SHAPIRO, 1953) so that the physiological production of lysolecithin inside the animal body is certainly possible. Indeed β-palmitoyl lysolecithin has recently been isolated from mammalian blood, liver, heart muscle and adrenal medulla (HAJDU, WEISS and TITUS, 1957), and it has been suggested that it may play an important physiological role. It must be remembered also that PIGHINI (1932) had earlier obtained an alcohol-soluble, ether-insoluble substance from brain, which, both from its solubility properties and biological actions, appeared to be a lysophosphatide.

SUMMARY

(1) A study has been made of the activity of various hydrolytic enzymes in brain homogenates 'cleared' with lysolecithin.

(2) It has been found that true and pseudo-cholinesterase, alkaline phosphatase, glycerylphosphorylcholine diesterase, proteinase and transaminase are all as active in the cleared as in control homogenates. Tributyrinase and acid phosphatase are also present, but are less active in the cleared preparations. Mono-amine oxidase is also less active.

(3) Lysolecithin has also been shown to cause a rapid release of both true and pseudo-cholinesterase from brain slices.

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ENZYMIC ACTIVITIES IN THE SPINAL CORD AFTER SCIATIC SECTION

ALKALINE AND ACID PHOSPHATASES, 5-NUCLEOTIDASE AND ATP-ase

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THE study of the metabolic changes taking place in the nervous system under conditions giving rise to chromatolytic degeneration has been stimulated by the development of microspectrophotometric techniques. The changes in nucleic acids and cell proteins during degenerative and regenerative processes, and during neuronal activity, have been investigated by CASPERSSON and HYDEN (1945) and by HYDEN (1947). There is considerable evidence that when the nerve cell is strongly stimulated or when the axon is cut, an abnormal consumption of ribonucleic acids and proteins takes place in the cytoplasm and, from a morphological point of view, chromatolytic changes occur; chromatolysis may thus be regarded as the cytological expression of a large reduction in the ribonucleic acid and protein concentration in the cell (HYDEN, 1955). The subject has recently been reviewed by EINARSON (1957).

In further investigations, attempts have been made to determine the alterations in enzymic activity associated with the nucleoprotein changes. The changes in phosphatase activity with which we are particularly concerned here have been studied (a) by histochemical methods and (b) by using chemical procedures. A review of the findings obtained by histochemical methods indicates that acid phosphatase activity increases in the motor neurones of the spinal cord (BODIAN and MELLORS, 1945; SCEVOLA, 1951) and spinal ganglia (SULKIN and KUNTZ, 1950) following section of the corresponding peripheral nerves. Under the same conditions there is a diminution in the alkaline phosphatase activity (SULKIN and KUNTZ, 1950; SCEVOLA, 1951). The results obtained under other conditions, when different methods were used to induce chromatolytic phenomena, show less agreement (SWANK and CAMMERMEYER, 1949; DELLA BEFFA, 1950; SULKIN and KUNTZ, 1950; SCEVOLA, 1951; SOULAIRAC and DESCLAUX, 1951; ORUNESU and ZUCCHI, 1954; DE RENZI and ROSSI, 1954). In the more recent experiments of BOZZETTI (1954) the effects of peripheral nerve section described above were not confirmed.

The results obtained by chemical techniques lead to different conclusions. BODIAN and FLEXNER (1946) showed in the anterior horns of the spinal cord of the macaque a mean decrease of 11 per cent in acid phosphatase activity after cutting the anterior roots. BOZZETTI (1953) studied the alkaline and acid phosphatases in the dog after sciatic section and found a reduction of enzymic activities in the spinal cord as well as in the brain and brachial plexus. Similar chemical procedures have been used also in a number of studies of phosphatase activity in transected peripheral

nerves (HEINZEN, 1947; BODIAN and FLEXNER, 1946; BOZZETTI, 1953). Particularly interesting for us are the investigations of HOLLINGER, ROSSITER and UPMAILS (1952) who, besides determining the changes in alkaline and acid phosphatases, studied the changes in 5-nucleotidase and ATP-ase activity in the two stumps of cut or crushed sciatic nerves in the guinea pig. Their findings show a 33 per cent increase in acid phosphatase activity, a 76 per cent decrease in alkaline phosphatase, a 50 per cent increase in 5-nucleotidase and a 20–60 per cent increase in ATP-ase.

The aim of the present work was to obtain further information about phosphatase changes in the central nervous system following peripheral nerve injury. For this purpose alkaline and acid phosphatase, 5-nucleotidase and Ca^{2+} activated ATP-ase have been assayed at various levels of the guinea pig spinal cord before and after bilateral sciatic nerve section.

METHODS

Seventy-five adult guinea pigs of both sexes were used. The sciatic nerves of both sides were cut high in the thigh under ether anaesthesia. To minimize the possibility of regeneration in the sectioned nerves, the proximal stump was retracted and sutured to the overlying muscle. The morphological aspects of the degenerative processes were observed at the various stages by histological methods in control animals, whose sciatic nerves had been sectioned on one side only.

From 3 to 60 days following sciatic section the animals were killed by decapitation and the spinal cord was immediately removed. After washing with water and removing the meninges, the lumbar and cervical enlargements and the middle part of the thoracic portion were cut and rapidly weighed at 0°C. The time needed from the moment the animal was killed was not more than 20 min. Three animals were used in each experiment and they were killed in the order (a) operated animal (b) normal animal, and (c) second operated animal.

After the third animal was killed and the spinal cord weighed, homogenates of the required dilution (1 : 3 w/v) were prepared in ice-cold distilled water, using an ice-cold Potter–Elvehjem type glass homogenizer. Tubes containing homogenate, substrate and buffer were placed in a water bath at 38° for 4 min for the ATP-ase, 10 min for the 5-nucleotidase and 60 min for the acid and alkaline phosphatase.

Incubation mixtures

Alkaline and acid phosphatase. 1 ml 0·03 M-β-glycerophosphate; 1 ml 3% homogenate; 1 ml 0·1 M-acetate–veronal buffer (pH 9·8 or 5·4).

5-*Nucleotidase.* 1 ml 0·003 M-adenosine-5-phosphate (SIGMA); 1 ml 3% homogenate; 1 ml 0·1 M-acetate–veronal buffer (pH 7·3).

Ca^{2+} *activated ATP-ase.* 1 ml 0·004 M-ATP (RICHTER) +0·2 M-$CaCl_2$; 1 ml 3% homogenate; 1 ml 0·1 M-acetate–veronal buffer (pH 7·3).

At the end of the incubation 1 ml of 10% tricloroacetic acid was added. The inorganic phosphate liberated by enzymic action was determined by BRIGGS' (1924) method using 1 ml of the deproteinized filtrate. The colorimetric readings were carried out with an e.e.l. apparatus at 640 mμ. The alkaline and acid phosphatase activities are expressed in milligrams of inorganic P liberated in 60 min from 100 g of fresh tissue. The values of 5-nucleotidase and ATP-ase are expressed in milligrams of inorganic P liberated in 10 min from 100 g of fresh tissue $\pm$ standard deviation.

RESULTS

Normal animals

Our experiments indicate that the enzymes studied have different activities at different levels of the spinal cord. Maximal values for acid and alkaline phosphatase and 5-nucleotidase are found in the lumbar enlargement and minimal values in the thoracic segment: the cervical enlargement gave intermediate values (Table 1).

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TABLE 1.—DISTRIBUTION OF ENZYMIC ACTIVITIES IN THE SPINAL CORD OF THE NORMAL GUINEA PIG
(the values are expressed as enzyme activities ±S.D.)

	Lumbar enlargement		Thoracic tract		Cervical enlargement	
	No. of exp.	Activity	No. of exp.	Activity	No. of exp.	Activity
Alkaline phosphatase	25	126·2 ± 13·8	25	77·9 ± 11·7	20	96·7 ± 17·5
Acid phosphatase	25	45·1 ± 6·5	25	35·6 ± 6·4	20	38·6 ± 5·7
5-Nucleotidase	24	39·2 ± 6·4	24	24 ± 6·7	19	26·6 ± 6·8
ATP-ase	22	365 ± 34	22	317 ± 43	17	311 ± 42

Operated animals

Alkaline phosphatase. After sciatic section a decrease in alkaline phosphatase activity at all levels of the spinal cord was observed. This decrease became evident in the first 6 days after operation; it reached its maximum between the ninth and the thirtieth day and persisted, with a slight increase, throughout the survival time (up to 60 days). The differences found between normal and operated animals at the three spinal cord levels examined were statistically significant ($P < 0{\cdot}001$) (Table 2).

TABLE 2.—ALKALINE PHOSPHATASE ACTIVITY IN THE SPINAL CORD OF GUINEA PIGS AFTER SECTION OF BOTH SCIATIC NERVES
(the changes are expressed as the difference per cent from the mean values given in Table 1)

Days after operation	Lumbar enlargement			Thoracic tract			Cervical enlargement		
	No. of exp.	Mean activity	Change (%)	No. of exp.	Mean activity	Change (%)	No. of exp.	Mean activity	Change (%)
3–6	6	103	−18·4	6	70·4	−9·6	6	81	−16·2
9–12	10	89·9	−28·8	10	47·3	−39·3	8	69·6	−28
15–18	10	85·6	−32·2	10	50·3	−35·4	8	71	−26·6
21–24	8	84·7	−32·9	8	45·4	−41·7	6	58·3	−39·7
27–30	8	85·7	−32·1	8	52·4	−32·7	6	65	−32·8
40–60	8	94·1	−25·4	8	56·4	−27·6	6	82·7	−14·5
3–60	50	89·8 ±16·1	−29 $P < 0{\cdot}001$	50	52·5 ±11·9	−32·5 $P < 0{\cdot}001$	40	71·1 ±19·3	−26·5 $P < 0{\cdot}001$

Acid phosphatase. In contrast to the behaviour of the alkaline phosphatase, any changes in acid phosphatase activity were small and inconstant. The differences found between normal and operated animals were not significant (Table 3).

5-Nucleotidase. The changes in activity of this enzyme were similar to those described for alkaline phosphatase in the lumbar and dorsal cord segments: sciatic

TABLE 3.—ACID PHOSPHATASE ACTIVITY IN THE SPINAL CORD OF GUINEA PIGS AFTER SECTION OF BOTH SCIATIC NERVES
(the changes are expressed as the difference per cent from the mean values given in Table 1)

Days after operation	Lumbar enlargement			Thoracic tract			Cervical enlargement		
	No. of exp.	Mean activity	Change (%)	No. of exp.	Mean activity	Change (%)	No. of exp.	Mean activity	Change (%)
3–6	6	50·6	+12·1	6	35·8	+0·5	6	33·8	−11·7
9–12	10	45·5	+0·9	10	34	−4·5	8	36·5	−5·5
15–18	10	41·4	−8·1	10	31·1	−12·6	8	34·3	−11·2
21–24	8	45·4	+0·6	8	35·2	−1·2	6	39·1	+1·4
27–30	8	45	—	8	35·4	−0·5	6	41·3	+7·1
40–60	8	44·8	−0·5	8	36·7	+3·2	6	37·6	−2·6
3–60	50	45·3 ±7·6	+0·4 $P > 0·05$	50	34·5 ±6·1	−3·1 $P > 0·05$	40	36·9 ±5·6	−4·5 $P > 0·05$

section clearly caused a decrease at these levels. The diminution of 5-nucleotidase activity was already evident during the first 6 days after operation and it reached a maximum between the ninth and twenty-fourth day: later on the values began to revert to normal. These changes are not paralleled by those observed in the cervical cord, where the reduction in enzymic activity was less pronounced than in the other

TABLE 4.—5-NUCLEOTIDASE ACTIVITY IN THE SPINAL CORD OF GUINEA PIG AFTER SECTION OF BOTH SCIATIC NERVES
(the changes are expressed as the difference per cent from the mean values given in Table 1)

Days after operation	Lumbar enlargement			Thoracic tract			Cervical enlargement		
	No. of exp.	Mean activity	Change (%)	No. of exp.	Mean activity	Change (%)	No. of exp.	Mean activity	Change (%)
3–6	6	35·2	−10·3	6	21·1	−11·9	6	25·5	−4·3
9–12	10	30·8	−21·5	10	17·4	−27·4	8	22·6	−14·9
15–18	10	27·8	−29·1	10	18·2	−24	8	25·1	−5·8
21–24	6	32·4	−17·4	6	18	−24·9	4	24	−9·8
27–30	8	35·8	−8·9	8	23·1	−3·8	6	23·5	−11·5
40–60	8	35·7	−8·8	8	22	−8·3	6	30·9	+16·5
3–60	48	32·4 ±6·7	−17·4 $P < 0·001$	48	19·7 ±5·3	−17·9 $P = 0·001$	38	24·8 ±5·2	−6·8 $P > 0·05$

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segments, and after the thirtieth day values higher than the normal were found. The statistical significance of the observed changes was apparently different at the three levels examined (Table 4).

ATP-ase. A reduction in activity of this enzyme was found at all the spinal cord levels examined, though it was not as great as that found for alkaline phosphatase and 5-nucleotidase. The values found did not show any particular variation with time. The statistical significance of the changes was small in the cervical cord ($P > 0{\cdot}05$) (Table 5).

TABLE 5.—Ca^{2+} ACTIVATED ATP-ASE IN THE SPINAL CORD OF GUINEA PIGS AFTER SECTION OF BOTH SCIATIC NERVES

(the changes are expressed as the difference per cent from the mean values given in Table 1)

Days after operation	Lumbar enlargement			Thoracic tract			Cervical enlargement		
	No. of exp.	Mean activity	Change (%)	No. of exp.	Mean activity	Change (%)	No. of exp.	Mean activity	Change (%)
3–6	6	327	−10·4	6	291	−8·3	6	296	−4·7
9–12	8	301	−17·6	8	267	−16	6	268	−13·7
15–18	8	339	−7·3	8	296	−6·7	6	293	−5·6
21–24	6	330	−9·7	6	278	−12·6	4	290	−6·8
27–30	8	341	−6·5	8	280	−11·8	6	291	−6·5
40–60	8	324	−11·2	8	277	−12·9	6	265	−14·7
3–60	44	327 ±51	−10·5	44	281 ±45	−11·5	34	283 ±50	−8·9
		$0{\cdot}01 > P > 0{\cdot}001$				$P > 0{\cdot}01$			$P > 0{\cdot}05$

DISCUSSION

The results obtained under the present experimental conditions indicate that the section of peripheral nerves markedly affects the enzymic *milieu* in the spinal cord. If a closer analysis of the results is made, some points arise which appear to deserve particular mention.

(1) In the guinea pig spinal cord, only three of the four enzymes studied were definitely affected by bilateral section of the sciatic nerves; these are the alkaline phosphatase, 5-nucleotidase and ATP-ase. No definite change appeared to occur in the acid phosphatase.

(2) All three of the enzymes affected showed a change in the same direction, i.e. a decrease in activity. The extent of this diminution was different for the three enzymes. The decrease in alkaline phosphatase activity was more than the decrease in 5-nucleotidase and still more than the decrease in ATP-ase.

(3) These changes occur in the whole spinal cord, having been found at the lumbar as well as at the thoracic and cervical levels. The degree of alteration at different cord levels is not the same for the three enzymes: the alkaline phosphatase activity was fairly uniformely reduced, but both the 5-nucleotidase and the ATP-ase were affected

more in the lower than in the upper (cervical) segments. It should be noted that the small significance found for these enzymic changes at the cervical level may be attributable in part to the smaller number of cases examined.

(4) All the enzymic changes described were detectable in the first few days after sciatic section and they increased later. With the passage of time, however, the changes in activity of the different enzymes did not proceed in parallel. The alkaline phosphatase and 5-nucleotidase reached their minimal levels in about 2 weeks, and later both of them, and particularly the 5-nucleotidase, showed a tendency to return to normal levels. The ATP-ase activity, on the other hand, showed a constant, although not very regular, diminution throughout the duration of the experiment (60 days).

At present, the only attempt to interpret the enzymic changes occurring in the spinal cord following peripheral denervation is that made by BODIAN and MELLORS (1945). According to these investigators an increase in acid phosphatase activity in the anterior horn motor cell after axon section can be demonstrated by histochemical techniques. They relate this enzymic change to the breakdown of nucleic acids, which occurs in the cell bodies during chromatolytic degeneration. Apart from the fact that, with the techniques we used, the acid phosphatase did not show any significant change, it seems unlikely that the changes in enzymic activity seen under the present conditions can be related to the chromatolytic phenomena. They are in fact clearly present also at spinal cord levels where, according to other workers and as shown in the control animals, no retrograde degeneration of the cell bodies occurs after sciatic nerve section. This conclusion is supported also by the observation of BOZZETTI (1954) previously mentioned.

The correct interpretation of the present results is not known, but it may be suggested that, following peripheral denervation, the central nervous system adapts to the new situation, and the enzymic changes might therefore be evidence of functional adaptation. Along this line of thought it would appear that further research might be useful to study the distribution of the enzymic changes within the central nervous system and to learn whether any relation can be established between the central structures showing enzymic changes and the peripheral lesion.

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SUMMARY

Alkaline and acid phosphatase, 5-nucleotidase and Ca^{2+} activated ATP-ase were determined in the spinal cord of the guinea pig (lumbar and cervical enlargement and dorsal tract) at times from 3 to 60 days after bilateral section of the sciatic nerves. Alkaline phosphatase activity was reduced to the greatest extent, followed by 5-nucleotidase and ATP-ase. Acid phosphatase activity did not change appreciably. These results were found in all segments of the cord examined and for the entire duration of the experiment in the case of the alkaline phosphatase and ATP-ase. For 5-nucleotidase the decrease was less marked in the cervical enlargement, and a return of values towards normal was observed in all segments at 24 days after the operation.

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BIOCHEMICAL AND PHYSIOLOGICAL DIFFERENTIATION DURING MORPHOGENESIS—XXIII

FURTHER OBSERVATIONS RELATING TO THE SYNTHESIS OF AMINO ACIDS AND PROTEINS BY THE CEREBRAL CORTEX AND LIVER OF THE MOUSE*

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THE observations presented here in part supplement previous ones concerning the newborn mouse (FLEXNER, FLEXNER and ROBERTS, 1958). In major part, however, they relate to the adult animal. Both cerebral cortex and liver have been studied to obtain estimates of the following quantities: (a) the rate at which blood supplies glutamic acid to these tissues; (b) the rate at which the carbon of glucose is incorporated into several non-essential amino acids of the tissues; (c) the rate at which certain essential amino acids are supplied to the tissues by the blood; and (d) the rate at which these amino acids in the tissues are incorporated into protein. It has been our interest to study the effect of growth and maturation on these quantities. The findings of GAITONDE and RICHTER (1956) with methionine and of LAJTHA, FURST, GERSTEIN and WAELSCH (1957) and LAJTHA, FURST and WAELSCH (1957) with lysine leave no doubt that amino acids are incorporated at a substantial rate into the proteins of the adult as well as the newborn brain.

METHODS

Glucose randomly labelled with ^{14}C was prepared from $Ba^{14}CO_3$, $^{14}CO_2$ being synthesized into sugars by *Canna* leaves (UDENFRIEND and GIBBS, 1949; PUTNAM and HASSID, 1952). The glucose was repeatedly chromatographed in phenol/water/ammonia (80 : 20 : 0·3 v/v) until radioactive contaminants (largely fructose) were reduced to less than 1 per cent; phenol was removed from the area of the chromatogram occupied by glucose by running the paper in *sec*-butanol/formic acid/water (70 : 10 : 30 v/v). With our counting arrangement, using a thin window Geiger tube, the glucose had a specific radioactivity of $3{\cdot}8 \times 10^4$ counts/min per mg carbon. Randomly labelled L-amino acids were obtained by growing *Chlorella pyrenoidosa* with $^{14}CO_2$ ($+CO_2$) as sole carbon source (ROBERTS, COWIE, ABELSON, BOLTON and BRITTEN, 1955); after a tenfold increase in cell mass the protein was separated, hydrolysed and two-dimensional ascending chromatograms run first in *sec*-butanol/formic acid/water followed by phenol/water/ammonia. After locating the amino acid spots with radioautograms, the spots were cut out, phenol removed by repeated extraction of the paper with equal parts of acetone and ether, the amino acids eluted and then tested for purity by two-dimensional chromatography in the solvents previously used. The amino acids had a specific radioactivity of $2{\cdot}5 \times 10^4$ counts/min per μg carbon.

The white mice were young adults, 60–70 days old, weighing 25–30 g. They had free access to food and water. Littermates were used for each experimental group. Equal volumes and, with two exceptions to be noted, equal amounts of radioactive material were injected subcutaneously over the flank in the animals of a group. After an appropriate interval the animals were sacrificed by disloca-

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tion of the cervical vertebrae. The chest was then opened and as much blood as possible was drawn from the heart into a small amount of heparin with needle and syringe. Samples of blood plasma of 0·2 to 0·4 ml obtained by centrifugation were then mixed with 4 ml of ice-cold 5% trichloroacetic acid (TCA). The cerebral cortex and liver were promptly removed and 50 mg of each was immediately homogenized in 4 ml of 5% ice-cold TCA.

The subsequent procedure has been fully discussed (ROBERTS *et al.*, 1955; FLEXNER *et al.*, 1958). In brief, after 30 min at 5° the homogenate was centrifuged and the supernatant fluid poured off. This is the cold TCA-soluble fraction and is assumed to contain the pool amino acids of the tissues. The precipitate was then sequentially treated for 30 min with 75% ethanol, equal parts of ether and 75% ethanol, each at 40–50°, and 5% TCA at 100°. The remaining precipitate was washed free of TCA, stirred in acidified ethanol and centrifuged; the precipitate was then stirred in ether and centrifuged. The precipitate is the TCA-insoluble or protein fraction. It was demonstrated to be free of glutathione.

This report deals with the cold TCA-soluble fraction and the TCA-insoluble fraction. The TCA from the cold TCA-soluble fraction was removed by shaking with 2 vols. of ether and discarding the ether for five successive times. This material was further fractionated using the cation exchange resin, Dowex 50-X8, in the acid form. In experiments with radioglucose, the fraction containing glucose and lactate which passed through the column was collected and subsequently chromatographed in *sec*-butanol/formic acid/water. Amino acids were eluted from the column with 6 vols. of 4 N-NH_4OH and the ammonia was then evaporated.

Protein precipitates from cortex and liver were hydrolysed in 0·3 ml 6 N-HCl at 106° for 16 hr. Samples of the protein hydrolysate corresponding to 20 mg cortex and 10–15 mg liver as well as the total amino acid portion of the cold TCA-soluble fraction were then each dried in a current of air and subsequently taken up in a drop of 30% H_2O_2 for transfer to the chromatogram paper. The fractions were run on two-dimensional, ascending chromatograms; first in *sec*-butanol/formic acid/water followed by phenol/water/ammonia. Radioautograms were made of the finished chromatograms and the radioactivity of the indicated spots counted directly from the paper. The chromatograms were then treated with ninhydrin for final identification of the spots.

Chromatograms were made of the amino acid portion of the cold TCA-soluble fraction of blood plasma, cortex and liver and of the hydrolysed protein of cortex and liver. In the experiment with labelled glucose, chromatograms were also made of the glucose–lactate portion of the cold TCA-soluble fraction from plasma, cortex and liver. The usual procedure was to count samples of fractions in cups (infinite thinness) and then to determine the proportionate radioactivity of components by counting the spots on the chromatograms. Cup values only are plotted in the graphs.

The concentrations of amino acids in the tissue pool and in hydrolysed tissue protein were determined in the following way: known amounts of radioactive amino acid were added to the protein hydrolysate derived from 15 mg liver and 20 mg cortex and, after elution from the column, to the pool amino acids derived from 200 mg tissue. After two-dimensional chromatography the spots were developed with dilute 0·01% ninhydrin, in acetone. Individual spots were then cut from the paper, and eluted with water, NaOH was added to give an alkaline reaction and the solutions taken down to dryness to rid them of ammonia. The residue was next taken up in water, neutralized with HCl and aliquots taken for counting and for determination of amino acids with ninhydrin (ROSEN, 1957).* Blanks were prepared from the chromatographic paper. The ninhydrin value was then corrected for losses in the procedure as indicated by the measured loss in radioactivity. Preliminary experiments with known amounts of amino acids indicated this to be a satisfactory procedure.

Samples of liver and cortex were taken after obtaining as much blood as possible from the heart. As in the newborn mouse, the contamination of labelled tissue protein by labelled blood protein in the adult does not appear to have been a significant source of error. After injection of labelled glutamic acid, the protein of whole blood never contained more than 1 per cent of the radioactivity of protein obtained from the same weight of liver at a corresponding time or 50 per cent of that from the same weight of cortex; after injection of the labelled essential amino acids, these ratios remained less than 5 per cent and 20 per cent. Inspection of the samples of cortex showed that they contained but little blood; this is substantiated by the quantitative observations of LAJTHA *et al.* (1957).

* The only satisfactory ninhydrin reagent we have found is that manufactured by Matheson, Coleman and Bell.

RESULTS

Amino acid composition of tissue pools and tissue protein

As shown in Table 1, differences between the composition of the amino acid pool of the newborn and adult liver and cortex do not follow a consistent pattern. In some instances the concentration of the amino acid is greater in the newborn than in the adult (CHRISTENSEN and STREICHER, 1948); in some, the adult values exceed the newborn (WAELSCH, 1951) and in others there is no apparent change with maturation. With respect to the amino acids which we have measured the composition of tissue protein is indistinguishable in newborn and in adult liver or cortex. The values for the amino acid composition of adult liver protein agree with those of SCHWEIGART, GUTHNECK, PRICE, MILLER and MILLER (1949).

Flux of amino acids from blood to tissue

Because of the presumed impermeability of the mature blood–brain barrier to glutamic acid, we have been interested in comparing the rates of transfer of this amino acid from blood to newborn and adult cortex; in both instances a comparison has also been made with rates of transfer of essential amino acids. Phenylalanine, leucine and *iso*leucine are not completely separated from one another by the solvents we have used for chromatography and have been used as a group to study behaviour of essential amino acids. Since their molar ratio is practically the same in the blood of the mouse (ALBRITTON, 1952) as in hydrolysate of *Chlorella* protein (ROBERTS *et al.*, 1955) their specific radioactivities in the blood after injection are substantially equivalent. In the experiments with labelled glutamic acid, $7{\cdot}0 \times 10^4$ counts/min per g of animal in 0·05 ml were injected subcutaneously; $4{\cdot}5 \times 10^4$ counts/min per g of animal of the essential amino acids were used in 0·05 ml.

The passage of radioactive amino acids from blood plasma to tissue pool to tissue protein in adult mice is shown in Figs. 1 and 2. Similar data obtained with newborn mice have been presented (FLEXNER *et al.*, 1958). Table 2 gives the calculated flux of amino acids from plasma to tissue; the previous data for the newborn mouse are included for comparison.

To calculate the flux from plasma to tissue it is assumed that:

$$F \times \bar{S}_p = \frac{\Delta T^*}{\Delta t}$$

where F is the flux, $\bar{S}_p$ is the average specific radioactivity of the amino acid in the plasma during the interval Δt, and ΔT^* is the increment of the total radioactivity of the amino acid of the tissue. This simplifying assumption, necessary for an amino acid with more than one source, neglects any reverse flow and thereby the flux may be underestimated except at the earliest times.

In calculating the flux of glutamic acid the additional assumption is made that it is the glutamic acid of the blood which supplies radioactivity to the glutamic acid and glutamine found in the tissue. This assumption seems reasonable since the flux is maximal at the earliest observation corresponding to the maximal radioactivity of plasma glutamic acid. In contrast plasma glutamine reaches its maximum at a considerably later time (see Fig. 1). If the radioactivity of plasma glutamine does

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TABLE 1.—AMINO ACID COMPOSITION OF TISSUE POOLS AND OF TISSUE PROTEIN

Amino acid	(μg in pool/100 mg tissue)				(mg/100 mg protein)			
	Liver		Cortex		Liver		Cortex	
	Newborn	Adult	Newborn	Adult	Newborn	Adult	Newborn	Adult
Aspartic	22 ± 3·5(5)	6·3 ± 1·0(6)	20 ± 1·6(6)	31 ± 3·0(7)	10(1)	10(1)	11(1)	11(1)
Glutamic	28 ± 3·6(5)	26 ± 1·9(6)	47 ± 4·2(6)	110 ± 5·8(7)	10(1)	9(1)	16(1)	17(1)
Glycine	13 ± 1·1(6)	7·0 ± 0·7(6)	10 ± 1·1(6)	6·3 ± 0·4(7)	4(1)	4(1)	3(1)	3(1)
Serine	9·0 ± 0·8(6)	4·8 ± 0·3(6)	7·0 ± 0·8(6)	4·4 ± 0·4(7)	4(1)	5(1)	4(1)	3(1)
Alanine	13 ± 1·2(6)	20 ± 1·7(6)	9·0 ± 1·2(6)	7·0 ± 0·9(7)	5(1)	6(1)	5(1)	5(1)
Ph. al. + Glutamine	8·1 ± 0·9(6)	6·0 ± 0·8(5)	6·2 ± 0·7(6)	2·5 ± 0·3(7)	24(1)	21(1)	14(1)	17(1)
	>10 ± 1·7(5)	>19 ± 4·0(4)	>13 ± 2·4(4)	>22 ± 4·8(6)				

Results on the amino acid pools are expressed in terms of the mean and its standard error. Number of samples are in parentheses. Since glutamine is lost on the column (MOORE and STEIN, 1954) the observed values are falsely low. The value for glutamic acid in protein includes glutamine converted to glutamic acid by acid hydrolysis. In this and following tables and graphs ph. al. + = phenylalanine + leucine + *iso*leucine.

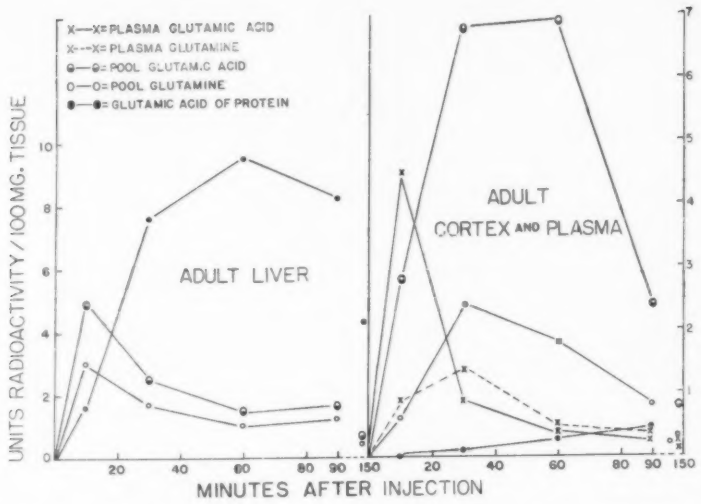

FIG. 1.—Radioactivity of glutamic acid and glutamine in blood plasma and in amino acid pools of cortex and liver, and of glutamic acid in proteins of cortex and liver following subcutaneous injection of L-glutamic acid randomly labelled with ^{14}C. In this and following graphs, 1 unit of radioactivity = 64 counts/min. The ordinate on the right is for cortex; that on the left, for liver. At 30 min the protein value of cortex = 0·1 unit radioactivity.

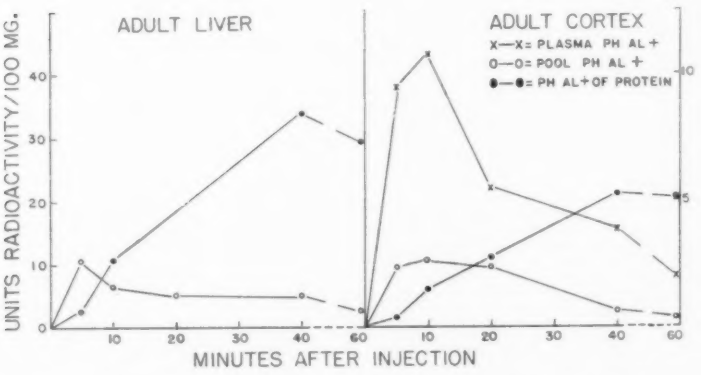

FIG. 2.—Radioactivity of phenylalanine + leucine + *iso*leucine (Ph. Al. +) in plasma, and in amino acid pools and proteins of cortex and the liver following subcutaneous injection of these amino acids randomly labelled with ^{14}C. The ordinate on the right is for cortex and plasma; that on the left, for liver.

in fact contribute appreciably to the radioactivity of the glutamic acid and glutamine of the tissue, then a correction should be made which would reduce the flux value for glutamic acid. This correction will be relatively small at early times because the ratio of radioactivity of the glutamine to that of glutamic acid is low.

The concentrations of amino acids in the plasma are those given by ALBRITTON (1952). For glutamic acid this is 3·3 mg/100 ml and for phenylalanine plus leucine plus *iso*leucine, 6·3 mg/100 ml.

TABLE 2.—FLUX OF AMINO ACIDS FROM PLASMA TO TISSUES

Amino acid	Newborn			Adult		
	(μg/100 mg tissue per hr)			(μg/100 mg tissue per hr)		
	t (min)	Cortex	Liver	*t* (min)	Cortex	Liver
Glutamic	10, 30, 60	—, 3, 4	15, 11, —	10, 30	29, 23	80, —
Ph. al. +	15, 30	28, 23	52, —	5	51	—

The molar concentration in blood plasma of phenylalanine plus leucine plus *iso*leucine is twice that of glutamic acid (ALBRITTON, 1952); for purposes of comparison half the observed rate of the phenylalanine group has been entered in the table. The times at which the animals were sacrificed after injection (*t*) correspond in order to the values in the following two columns. — indicates data which do not permit calculation of rate at corresponding time.

Synthesis of amino acids from glucose

Following the injection of labelled glucose, we have estimated the total amount of tissue amino acid carbon derived from plasma glucose/unit time per unit weight of tissue and have measured the distribution of this carbon among the labelled amino acids. We have further been interested in comparing the relative importance of plasma glutamic acid and glucose as sources of tissue glutamic acid. Finally, we have been concerned with the possibility that essential amino acids may be labelled with glucose carbon as has been observed *in vivo* in the newborn liver and brain (WINZLER, MOLDAVE, RAFELSON and PEARSON, 1952; FLEXNER *et al.*, 1958).

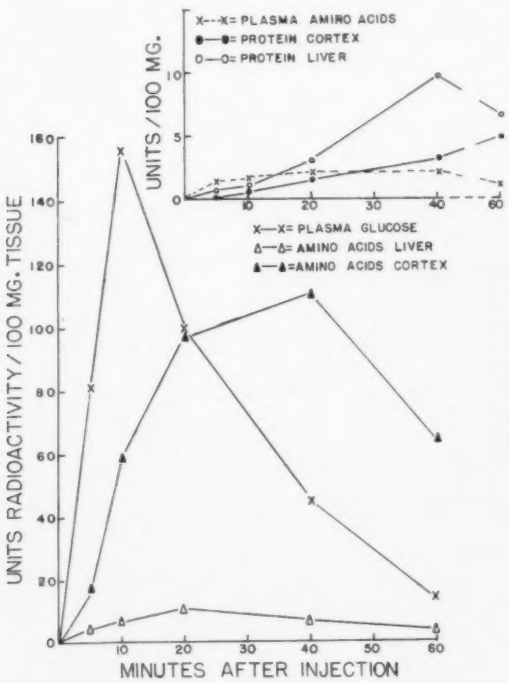

FIG. 3.—Radioactivity of plasma glucose and of total amino acids of pool/100 mg tissue following subcutaneous injection of glucose randomly labelled with ^{14}C. In the insert, total radioactivity of protein/100 mg tissue and of plasma amino acids/100 mg plasma. Chromatograms of the plasma at 40 and 60 min showed that 8 per cent of the radioactivity of the amino acids was derived from glutamic acid and 8 per cent from glutamine.

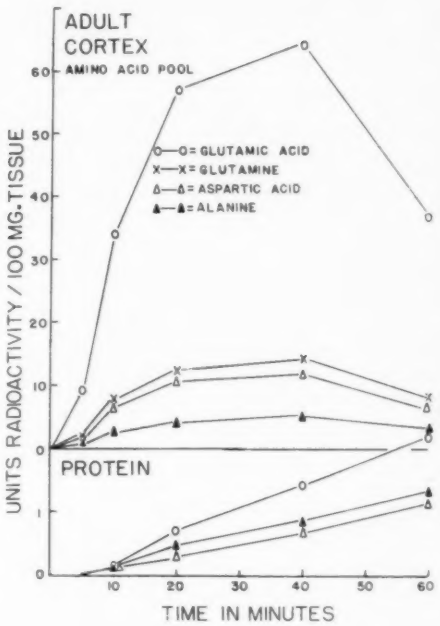

FIG. 4.—Radioactivities of individual amino acids of amino acid pool and protein hydrolysate of cortex following injection of glucose randomly labelled with ^{14}C. At 10 min the protein values for aspartic acid = 0·08 unit radioactivity; for alanine = 0·10; for glutamic acid = 0·16. In addition to the amino acids of the figure the following in the cold TCA-soluble fraction were found to contain ^{14}C: γ-amino butyric acid (values midway between aspartic acid and glutamine) and traces of proline; in protein, in amounts too low for counting, were serine, glycine and proline.

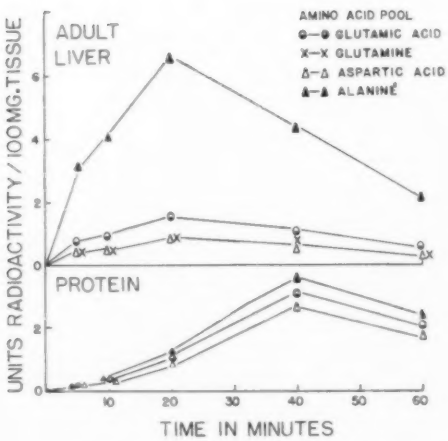

FIG. 5.—Radioactivities of individual amino acids in amino acid pool and of protein hydrolysate of liver following subcutaneous injection of glucose randomly labelled with ^{14}C. At 5 and 10 min, respectively, the protein values for aspartic acid = 0·11 and 0·22; for alanine = 0·15 and 0·30; for glutamic acid = 0·13 and 0·26. In addition to the amino acids of the figure the following were found to contain ^{14}C in amounts too low for counting: in the cold TCA-soluble fraction, cysteic acid, serine and glycine; in protein, serine, glycine and proline.

Animals sacrificed at 5, 10 and 20 min after injection received $9{\cdot}0 \times 10^4$ counts/min per g of animal of labelled glucose in 0·05 ml; animals sacrificed at 40 and 60 min received $1{\cdot}6 \times 10^6$ counts/min per g in 0·05 ml. Values obtained from the tissues of these last two animals were correspondingly reduced for Figs. 3, 4 and 5.

The radioactivity of glucose and lactate in the glucose–lactate portion of the cold TCA-soluble fraction was measured after chromatography. Of the total radioactivity in glucose and lactate, glucose contained the following percentages: for plasma, 80–90 per cent; for cortex, 20–30 per cent; for liver, 89–92 per cent.

The rates of Table 3 have been derived from the observations of Figs. 3, 4 and 5 and from our previously presented observations on the newborn. The calculations

are similar to those described above for amino acid flux. The concentration of glucose in the plasma was found to be 100 mg/100 ml both in the adult and in the newborn mouse. These rates may be minimal since plasma glucose is not the immediate precursor of the labelled amino acids; it is to be noted in the adult, however, that rates obtained soon after injection of labelled glucose are equal to or higher than subsequent ones indicating that intermediate pools quickly reach their maximum specific radioactivities.

TABLE 3.—APPARENT RATE OF INCORPORATION OF GLUCOSE CARBON INTO AMINO ACIDS (TOTAL) AND SPECIFICALLY INTO GLUTAMIC ACID PLUS GLUTAMINE (GLUTAMIC +)

Amino acid	Newborn			Adult		
	(μg C/100 mg tissue per hr)			(μg C/100 mg tissue per hr)		
	t (min)	Cortex	Liver	t (min)	Cortex	Liver
Total	17, 60	11, 19	13, 14	5, 10, 20	210, 190, 120	72, 25, 17
Glutamic +	17, 60	8, 13	3, 6	5, 10, 20	140, 135, 85	16, 5, 3

The times at which the animals were sacrificed (t) correspond in order to the values in the following two columns. Late values may be low because reverse flow has been neglected in calculating flux.

In the newborn animal the rates at which glucose supplies carbon for synthesis of non-essential amino acids and specifically for glutamic acid is little or no different in cortex and liver. In the adult cortex the rate at which carbon of glucose is incorporated into non-essential amino acids and specifically into glutamic acid far exceeds that both in the newborn cortex and in the adult liver.

To compare the relative importance of plasma glutamic acid and glucose as sources of glutamic acid in the tissues, the flux values for glutamic acid given in Table 2 must be multiplied by 0·4 to convert them to carbon of glutamic acid. Comparison of these values with those of Table 3 indicate that glucose supplies approximately 10 times as much carbon to glutamic acid of both newborn and adult cortex as does plasma glutamic acid. By contrast, the two sources are of about equal importance in both newborn and adult liver. It is evident from Fig. 1 and from the plasma level of labelled glutamic acid and glutamine given in Fig. 3 that, as in the newborn, the amount of ^{14}C derived secondarily by either cortex or liver from these amino acids in the blood is inconsequential in the experiment with labelled glucose.

No evidence has been found for labelling of essential amino acids from glucose in the adult animal.

Rates of incorporation of amino acids into protein

The estimated rates at which the amino acids which we have studied are incorporated into protein are given in Table 4. These rates for the newborn have been derived from our data previously presented; those for the adult come from the observations of Figs. 1, 2, 4 and 5. Two points call for comment: first, both radioactive glutamic acid and glutamine are precursors of the glutamic acid isolated from protein after acid hydrolysis. To derive a rate for incorporation of glutamic acid

plus glutamine into protein it is necessary that the specific radioactivities of the two amino acids in the pool be equal. We have not determined the specific radioactivites of the amino acids in individual experiments but have compared the ratios of radioactive glutamic acid to radioactive glutamine in all experiments with the ratio of their mean pool concentrations as given in Table 1. Some glutamine, as has been remarked, is lost on the column but with a constant technical procedure it can be assumed that the loss is relatively constant (MOORE and STEIN, 1954). In practically all experiments except for one series the specific radioactivities of the two amino acids were indistinguishable (Table 1, Figs. 1, 4 and 5). The exception was the cortex of the series of adult mice injected with glutamic acid; in this instance the specific radioactivity of glutamic acid averaged 65 per cent of that of glutamine (Fig. 1).

The second point concerns the use of phenylalanine, leucine and *iso*leucine as a group rather than individually. This procedure is believed to have led to no important error and had the advantage of placing additional radioactivity at our disposal. As has been said, the molar ratios of these amino acids in the hydrolysate of *Chlorella* protein, used as their source, is practically the same as in the blood of the mouse. Since they are essential amino acids there are no additional sources to distort their specific activities in the pool. We could detect no differences in their specific radioactivities in protein of cortex or liver.

The data of Table 4 lead to the following conclusions:

(1) The apparent rate of incorporation of an amino acid into the protein of the newborn tissues may be greater than, equal to or less than the adult. In the newborn liver the essential amino acids and perhaps aspartic acid are incorporated at a higher rate than in the adult; by contrast, the rate of incorporation of glutamic acid and glutamine in the adult far exceeds that in the newborn; the rates for alanine are indistinguishable at the two stages of development. In the newborn cortex the essential amino acids and alanine are incorporated at a greater rate than in the adult, while aspartic acid, glutamic acid and glutamine appear much the same at the two ages.

(2) The ratio of apparent rates of incorporation of amino acids into protein may differ substantially from the ratio of concentration of these amino acids in the protein. Table 5 gives for each of the amino acids, relative to the phenylalanine group, the concentration ratio in protein and ratio of apparent rate of incorporation into protein. The two ratios for all amino acids agree best in the adult cortex; lack of correspondence is particularly evident in the newborn cortex for aspartic acid, alanine and glutamic acid/glutamine; in the newborn liver for alanine and glutamic acid/glutamine; and in the adult liver for glutamic acid/glutamine.

DISCUSSION

Because of the failure to increase the concentration of glutamic acid in the adult brain by large increases in its concentration in the blood (SCHWERIN, BESSMAN and WAELSCH, 1950; HIMWICH, PETERSEN and ALLEN, 1957), it has generally been assumed that, unlike the immature blood–brain barrier, the mature barrier is impermeable to the amino acid. LAJTHA, quoted by WAELSCH (1958), has shown, however, that labelled glutamic acid in the adult is transferred from blood to brain at rates only slightly less than we have observed in the adult cortex. Indeed our results indicate that flux of glutamic acid from blood to cortex is approximately 10 times greater in

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TABLE 4.—FLUX OF AMINO ACIDS FROM POOL TO PROTEIN

Amino acid	t (min)	(μg to protein/100 mg tissue per hr) Newborn		t (min)	(μg to protein/100 mg tissue per hr) Adult	
		Cortex	Liver		Cortex	Liver
Ph. al. +	15, 30, 60	27, 23, 24	132, 135, 100	5, 10, 20, 40	11, 12, 10, 11	(35), 55, —, 54
Glutamic +	10, 30, 60, 240	—, —, 5, 4	—, (4), 15, 10	10, 30, 60, 90	—, 7, 6, 6	110, 116, 100, —
Glutamic +*	17, 60, 120, 240	—, 11, 9, 8	—, 29, 23, —	5, 10, 20, 40, 60	—, 10, 8, 5, 5	156, 104, 120, 160, —
Aspartic*	17, 60, 120, 240	—, 7, 5, 5	—, 86, 55, —	5, 10, 20, 40, 60	—, 7, 5, 4, 4	45, 27, 33, 45, —
Alanine*	17, 60, 120, 240	—, 25, 17, 12	—, 18, 15, —	5, 10, 20, 40, 60	—, 5, 4, 3, 3	24, 14, 17, 22, —
Serine*	17, 60, 120, 240	—, 5, 3, 4	—, 23, 14, —			
Glycine*	17, 60, 120, 240		—, 25, 16, —			

* Starred amino acids are those labelled in the experiments with radioglucose. The times at which the animals were sacrificed after injection (t) correspond in order to the values in the following two columns. — indicates data which do not permit calculation of rate of corresponding time. Rates have been calculated from the time preceding the first appearance of measurable amounts of radioactivity in the amino acids of protein, e.g. in Fig. 4 from $t = 5$ min. Glutamine + = glutamine acid plus glutamine.

the adult than in the newborn; and in the adult, on the basis of equimolar concentrations in the blood, is half that of the essential amino acids we have studied.

In spite of the availability of blood glutamic acid to the brain, glucose appears to supply by synthesis *in situ* at least 10 times as much glutamic acid in both newborn and adult cortex as is derived by flux of the amino acid from the blood. That the central nervous system may be unique in this regard is suggested by the contrasting results on the liver which indicate that in this organ the two sources supply approximately equal amounts of glutamic acid.

In vivo experiments on the newborn mouse gave evidence of incorporation of glucose carbon into certain essential amino acids of both brain and liver (WINZLER *et al.*, 1952; FLEXNER *et al.*, 1958). In the adult brain *in vitro* WINZLER and his collaborators found incorporation of glucose carbon into valine and arginine. Our observations made *in vivo* on the adult mouse failed to substantiate these *in vitro* results and, as pointed out by WINZLER and his collaborators, emphasize the caution necessary in this field in transferring findings of *in vitro* experiments to the living animal.

We have used a series of amino acids in an effort to gain insight into the nature of the process of incorporation of amino acids into protein of cortex and liver. Three principal possibilities must be considered: (a) that an amino acid is incorporated into protein solely as a result of synthesis of the complete molecule; (b) that incorporation is the result of exchange reactions involving individual amino acids without

TABLE 5.—CONCENTRATION RATIOS OF AMINO ACIDS IN TISSUE PROTEIN (C.R.) AND RATIOS OF RATES OF INCORPORATION OF THESE AMINO ACIDS INTO TISSUE PROTEIN (R.R.). ALL REFERRED TO PHENYLALANINE, LEUCINE PLUS *iso*LEUCINE

Amino acid	Newborn				Adult			
	Cortex		Liver		Cortex		Liver	
	C.R.	R.R.	C.R.	R.R.	C.R.	R.R.	C.R.	R.R.
Aspartic*	0·8	0·3, 0·2	0·4	0·7 0·6	0·7	0·6, 0·5	0·5	0·8, 0·7
Glutamic+	1·1	0·2, 0·2	0·4	0·1, 0·1	1·0	0·6, 0·6	0·4	2·0, 2·0
Glutamic+*	1·1	0·4, 0·4	0·4	0·2, 0·2	1·0	0·9, 0·6	0·4	3·0, 2·5
Glycine*			0·2	0·2, 0·2				
Serine*	0·3	0·2, 0·2	0·2	0·2, 0·2				
Alanine*	0·4	0·9, 0·7	0·2	0·1, 0·1	0·3	0·5, 0·4	0·3	0·4, 0·4,

* The first rate ratio is that derived from the first observation after injection of labelled material; the second, is the average of all observations. The two rates in parenthesis in Table 4 have not been used. Starred amino acids are those labelled in the experiments with radioglucose. Glutamic + = glutamic acid plus glutamine.

complete degradation of the molecule, for which evidence can be found in other systems (GALE and FOLKES, 1955; HALVORSON, FRY and SCHWEMMIN, 1955; VAUGHAN and ANFINSEN, 1954; EAGLE, PIEZ and FLEISCHMAN, 1957); or (c) that there is a combination of these two processes. If synthesis were the only process involved, the rate of incorporation of any amino acid should be proportional to its concentration in the protein.

The data of Table 5 show deviations from this rule both in adult and newborn

tissues. These results suggest that exchange may be an important process. They do not provide a complete proof of exchange. It is quite possible that certain proteins of the tissue which are synthesized and degraded rapidly account for most of the incorporation whereas the average composition is determined by other proteins. Exploratory experiments using ion-exchange columns to separate out individual proteins have already shown that different proteins do in fact have different rates of incorporation of amino acids. It seems unlikely, however, that these differences will be sufficient to account for the observations of Table 5.

Additonal evidence for exchange comes from the finding that the apparent rate of incorporation of an amino acid in growing tissue may be greater than, equal to or less than in the adult in spite of the close correspondence in the amino acid composition of the protein at the two ages. GAITONDE and RICHTER (1956) have found that the young brain incorporates methionine more rapidly than the old; on the other hand, LAJTHA *et al.* (1957) found no clear difference in the rate of incorporation of lysine into the protein of young and adult liver.

Most of our experiments were carried out for a long enough period to observe the time after injection when the radioactivity of the tissue protein reached a plateau or declined. This time in newborn tissue was invariably longer than in the adult. The observations consequently support the thesis of RITTENBURG, SPROUL and SHEMIN (1948) that during growth there may be a reduced rate of protein breakdown though it is not clear how much of this breakdown is due to true degradation and how much to exchange.

SUMMARY

In contrast to the widely held view that, unlike the immature blood–brain barrier, the mature barrier is impermeable to glutamic acid, the observed flux of glutamic acid from blood to cortex is approximately 10 times greater in the adult than in the newborn. Nevertheless, glucose appears to supply by synthesis *in situ* at least 10 times as much glutamic acid in both newborn and adult cortex as is derived by flux of the amino acid from the blood.

Unlike the newborn, no incorporation of glucose carbon into essential amino acids was observed *in vivo* in the adult cortex or liver.

The apparent rate of incorporation of an amino acid into protein of newborn tissue may be greater than, equal to or less than that found in the adult. Moreover, the concentration ratios of amino acids in tissue protein is not consistently paralleled by the ratios of apparent rates of incorporation of these amino acids into tissue protein. For these reasons it is tentatively suggested that exchange reactions account in part for incorporation of amino acids into protein.

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Papers to be published in future issues

W. K. JORDAN, R. MARCH, O. B. HOUCHIN and E. POPP: Intracellular partition of purine deaminases in rodent brain.

H. UTENA, T. EZOE, N. KATO and H. HADA: Effects of chronic administration of methamphetamine in enzymic patterns in brain tissue.

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THE PROTEOLIPID NATURE OF THE NEUROKERATIN NETWORK OF MYELIN*

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EWALD and KÜHNE (1877) described the presence of a substance in the central nervous system, retina and peripheral nerves which, like keratin of epidermis, resisted solution in lipid solvents, dilute acids and dilute bases, and was not digested by gastric and pancreatic juices. In transverse sections of peripheral nerve and of white matter, this material, which they designated neurokeratin, appeared as pairs of concentric hollow tubes joined by bridges of the same material. The irregular network of stainable substance which persists in myelin sheaths following fixation and extraction of lipids has since been called 'neurokeratin', although in a histological rather than a biochemical sense. The investigations of HOERR (1936), SCHÜMMELFEDER (1950) and others (reviewed by STOECKENIUS and ZEIGER, 1956) indicate clearly that the neurokeratin network of myelin sheaths seen in ordinary histological preparations is a precipitation artifact derived evidently from protein present in the myelin sheath.

Because various biochemical preparations of neurokeratin have shown considerable differences in elementary composition, some doubt has existed as to whether neurokeratin is a real constituent of the nervous system or an artifact derived from other proteins during its preparation (reviewed by BLOCK, 1951). Studies carried out by FOLCH and his co-workers in recent years have clarified this problem. The neurokeratin prepared by the classical method is evidently a mixture derived from the protein moiety of proteolipid (FOLCH and LEES, 1951) and a trypsin-resistant protein residue (TRPR) which contains inositol phosphatide (LEBARON and FOLCH, 1956); both of these proteins resist tryptic and peptic digestion. Proteolipid protein comprises 2 per cent of white matter and 0·6 per cent of grey matter; the TRPR is evenly distributed between white and grey matter and constitutes 0·38 per cent of brain (LEBARON and FOLCH, 1956; FOLCH and LEBARON, 1957). These data suggest that proteolipid protein would contribute more to neurokeratin as prepared by the classical method than the TRPR.

Proteolipid first appears in the developing brain when myelination commences and increases in amount as myelination proceeds (FOLCH, 1955). This and the high concentration of proteolipid in white matter as compared with grey matter imply that proteolipid is a constituent of the myelin sheath. The TRPR has been suggested as

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a constituent of the myelin sheath on the ground that protein-bound inositide phosphorus, presumed to be a component of the TRPR, diminishes and increases *pari passu* with lipid phosphorus during Wallerian degeneration and regeneration respectively (LOGAN, MANNELL and ROSSITER, 1952). Neither of these inferences appears to have direct corroboration.

The solubility of proteolipid and the insolubility of the TRPR in chloroform-methanol afford an opportunity to investigate this problem by histological methods. These solubility differences have been employed in the present investigation. The results of this study show clearly that the neurokeratin network of myelin sheaths is derived principally, if not entirely, from proteolipid protein. Inferentially, the lipid moiety of proteolipid is also a myelin sheath constituent. Proteolipid protein appears to be endowed with striking tinctorial properties. Indeed, the effectiveness of the WEIL (1945) and KLÜVER and BARRERA (1953) methods in staining myelin can be attributed to the affinity of proteolipid protein for these dyes. Furthermore, the preliminary extraction of proteolipid from neural tissue renders axones stainable by a number of methods. This finding may be of value in the development of improved methods for the staining of axones and other neural structures.

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MATERIALS AND METHODS

The spinal cord and peripheral nerve of adult cats were employed in this investigation. Small blocks of tissue were fixed by immersion in 10% neutral formalin for one or more days. Adjacent blocks were soaked in chloroform–methanol (2 : 1 v/v) for 24–48 hr prior to fixation in 10% formalin. Following fixation, the tissue blocks were dehydrated in graded concentrations of ethanol, cleared in xylene and imbedded in paraffin. Sections were cut at 15 μ.

Proteolipid was prepared from fresh feline spinal cord by the method of FOLCH and LEES (1951) and dissolved in chloroform–methanol. The solution was placed on glass slides with a medicine dropper, the solvent evaporating with the aid of gentle heat. The slides were then soaked in xylene or chloroform–methanol to extract the lipids, leaving a film of denatured proteolipid protein on the slides.

Sections of formalin-fixed and chloroform–methanol extracted, formalin-fixed tissues were mounted on the same slides to ensure identical treatment. They were soaked in xylene to remove the paraffin and residual lipids and dehydrated through graded concentrations of ethanol prior to staining. In some instances, sections were further extracted in chloroform–methanol without altering the results. Tissue sections and proteolipid protein films were stained with the WEIL method (1945) and the KLÜVER–BARRERA method (1953) for myelin. Thionin, a basic dye, was used at pH 7·4 (0·5% thionin in 0·1 M-sodium acetate for 1½ hr followed by brief differentiation in 75% ethanol) to demonstrate the acidic groups of protein. Fast green, an acid dye, was employed at pH 0·9 (0·1% fast green in 0·1 N-HCl for 1 hr followed by differentiation in 75% ethanol) to show the basic groups of protein. The periodic acid–Schiff (PAS) method was also used. Direct staining of unoxidized sections with the Schiff reagent and acetylation of sections for 2 hr at 37°C (LILLIE, 1954) prior to the PAS procedure served as controls.

RESULTS

Formalin-fixed neural tissues

The WEIL method stained white matter of spinal cord black, grey matter a light brown. Microscopic examination revealed that the neurokeratin network was stained an intense black. In transverse sections of white matter, the neurokeratin network in large myelin sheaths typically had the appearance of a deeply stained pair of concentric or eccentric hollow tubes. The inner tube enveloped a poorly stained axone; the outer tube formed the external limit of the myelin sheath. Slender trabeculae of

neurokeratin were present between the inner and outer tubes. The trabeculae were radially, concentrically or irregularly disposed and constituted a relatively small fraction of the available space between the inner and outer tubes. Areas of white matter which contained numerous small, poorly myelinated fibres nevertheless contained much neurokeratin, judging from the intensity of staining. In longitudinal sections of white matter, few structural features of the neurokeratin network were discernible. It took the form of a vacuolated black-stained reticulum which gave slight indication of the linear arrangement of the nerve fibres. Axones were infrequently seen. In favourable instances, deeply stained trabeculae of irregular shape could be followed from inner to outer tubes separated by large unstained spaces (Figs. 1 and 3).

The myelin sheaths of peripheral nerve stained by the Weil method differed in a striking manner from the myelin sheaths in the spinal cord. Peripheral nerve took a heavier overall stain than white matter. This was readily seen in nerve roots where an abrupt transition of myelin from the central to the peripheral type took place a fraction of a millimetre from the neuraxis. This junction was also marked by the replacement of glia with Schwann cells. A similar junction was present in the sensory root of the trigeminal nerve 1–2 mm from the pons. In cross-sections of peripheral nerve, the neurokeratin usually took the form of black-stained clumps or bars radiating from the axone like the spokes of a wheel; less often it was concentrically disposed. There was more stainable neurokeratin and less unstained space in peripheral myelin sheaths than in central myelin sheaths. In longitudinal section, the neurokeratin was densely stained and assumed the form of a linear series of cone-tipped cylindrical or infundibular structures provided with vacuoles of various sizes and shapes. (Figs. 5, 7, 13 and 16).

The KLÜVER–BARRERA method stained white matter a blue–violet and grey matter a darker violet. The neurokeratin network of myelin sheaths in the white matter of spinal cord and in peripheral nerve stained a deep blue–violet but otherwise had the same appearance as neurokeratin in tissues stained by the WEIL method. Axons were not conspicuous. When Luxol fast blue was employed without cresyl violet as a counterstain, the neurokeratin stained light blue–green rather than blue–violet.

Thionin at pH 7·4 stained formalin-fixed neural tissues in a manner closely resembling the KLÜVER–BARRERA stain. White matter was a blue–violet, grey matter a deeper blue–violet, and peripheral nerve an intense violet. On microscopic examination, the neurokeratin was stained deep blue–purple and had the appearance already described. In white matter and peripheral nerve, thionin was almost as brilliant and selective a stain for myelin as the WEIL and the KLÜVER–BARRERA methods; in grey matter it did not stain myelin selectively. Axones in white matter and in peripheral nerve were stained light blue. However, they were usually obscured by the enveloping neurokeratin.

Fast green at pH 0·9 stained neural tissue a bright green, grey matter being more intensely stained than white matter. The neurokeratin network was stained in both central and peripheral myelin sheaths. However, fast green did not stain neurokeratin as intensely or selectively as the WEIL, KLÜVER–BARRERA and thionin methods. Axons were more intensely coloured than neurokeratin, although somewhat obscured by the latter.

The periodic acid–Schiff (PAS) method stained neurokeratin a definite pink.

Axons were generally hidden by the neurokeratin, but when seen were only faintly stained. Blood vessels, meninges and connective tissues in peripheral nerve stained a deeper pink than neurokeratin. Exposure of tissue sections not oxidized by periodic acid to the Schiff reagent resulted in a markedly fainter coloration of all structures. Sections which were acetylated before the PAS procedure gave only a trace reaction (Fig. 9).

Chloroform–methanol extracted neural tissues

Neural tissues soaked in chloroform–methanol prior to fixation in formalin underwent a moderate degree of shrinkage. Sections cut from tissues treated in this manner exhibited a striking alteration in staining.

With the WEIL method white matter of spinal cord no longer stained black but light brown. Microscopic examination revealed a total absence of the neurokeratin network in the myelin sheaths. Instead, the axons stood out conspicuously by virtue of their moderate staining which was offset by the surrounding halos of empty space. In the absence of the obscuring neurokeratin, even the very fine, poorly myelinated axons were plainly visible. Axons, both large and small, were strikingly evident in longitudinal sections of white matter. Indeed, these preparations compared favourably with sections stained specifically for axis cylinders by the Bodian method (Figs. 2, 4 and 14).

With the KLÜVER–BARRERA stain, white matter of spinal cord was stained light blue instead of deep blue–violet; no neurokeratin was present. Instead, the axons were distinctly stained in both transverse and longitudinal sections of white matter (Fig. 18).

With thionin at pH 7·4, white matter of spinal cord was stained light blue instead of deep blue–violet. Microscopic examination revealed no neurokeratin network in myelin sheaths. Axons were stained blue.

With fast green at pH 0·9, white matter of spinal cord showed little reduction in staining on gross examination. However, microscopic examination of these sections again revealed an absence of the neurokeratin network. Axons were stained a deep green.

With the PAS procedure, a distinct reduction in coloration of white matter was evident in white matter of spinal cord. The neurokeratin network was not present. Axons were faintly stained. However, blood vessels and the dura were deeply stained. The direct Schiff reaction showed a trace of colour in white matter. Acetylated sections gave no reaction at all upon application of the PAS procedure.

All five staining methods revealed a gross reduction in coloration of peripheral nerve. In transverse sections, no neurokeratin staining was evident. Axons were more darkly stained and were surrounded by virtually empty spaces which ordinarily contained the neurokeratin network. Schwann cell cytoplasm, not seen in formalin-fixed sections, enveloped each periaxonal space in the form of a hollow tube several microns thick. Many of the axons were oriented longitudinally or obliquely in the transverse sections; this misplacement of axons apparently occurred during sectioning as a result of the loss of support ordinarily afforded by the neurokeratin network. The appearance of peripheral nerve in longitudinal section was also altered. The infundibular and cylindrical segments of neurokeratin were conspicuously absent. However, axons were not as outstanding in longitudinal sections of peripheral nerve

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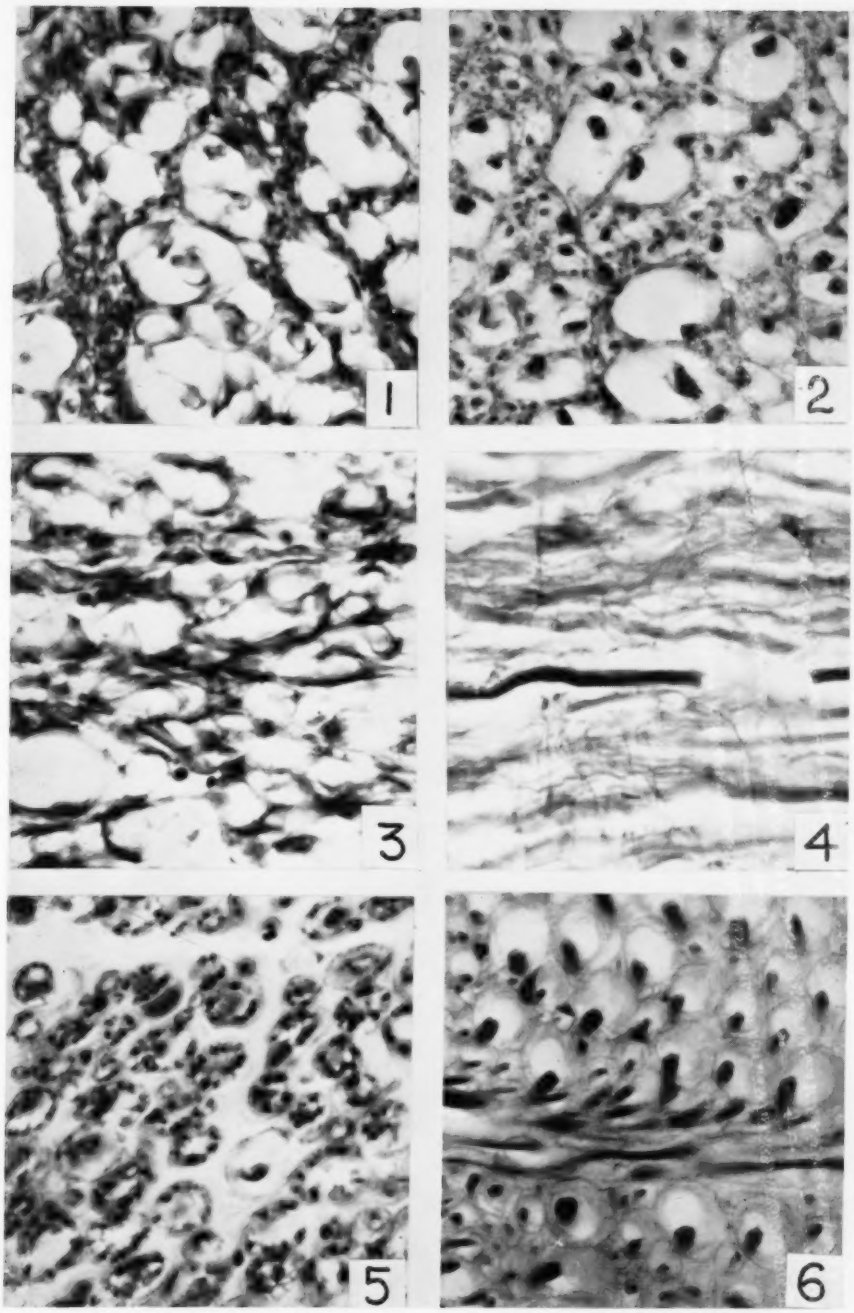

FIGS. 1–6.—Photomicrographs of neural tissue sections stained by the WEIL method. ×600.

FIG. 1.—Formalin-fixed white matter, transverse section. The neurokeratin reticulum, the protein remnant of the myelin sheaths, is stained black. Most axons are poorly stained and are encircled by neurokeratin. The empty spaces represent sites of dissolved myelin lipides.

FIG. 2.—Chloroform–methanol extracted white matter, transverse section. The neurokeratin network is absent. Axons are deeply stained and are surrounded by empty spaces of varying sizes which were formerly occupied by myelin sheaths.

FIG. 3.—Formalin-fixed white matter, longitudinal section.

FIG. 4.—Chloroform–methanol extracted white matter, longitudinal section. In the absence of the obscuring neurokeratin the axons are clearly delineated.

FIG. 5.—Formalin-fixed peripheral nerve, transverse section. The radiate pattern of black-stained neurokeratin is evident. Axons are poorly stained.

FIG. 6.—Chloroform–methanol extracted peripheral nerve, transverse section. The neurokeratin reticulum is gone and in its place is the empty space previously occupied by myelin sheaths. Axons are deeply stained.

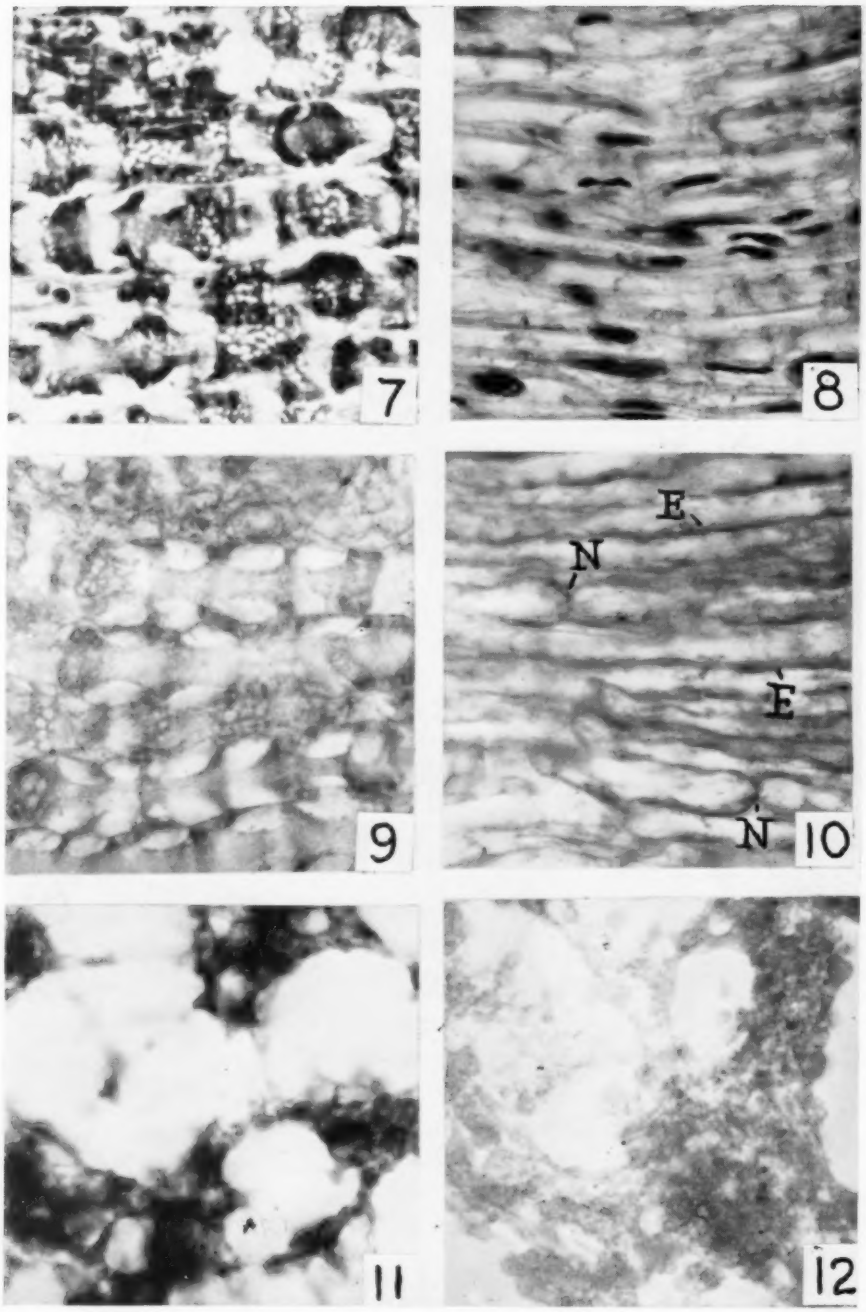

FIG. 7.—Formalin-fixed peripheral nerve, longitudinal section. The cylindrical and funnel-shaped segments of intensely stained neurokeratin are evident. Note the presence of vacuoles in the neurokeratin network. WEIL stain. ×600.

FIG. 8.—Chloroform–methanol extracted peripheral nerve, longitudinal section. The neurokeratin reticulum is not seen. Some axons are seen as well as Schwann cell nuclei. WEIL stain. ×600.

FIG. 9.—Formalin-fixed peripheral nerve, longitudinal section. The cylindrical and funnel-shaped segments of neurokeratin are vacuolated and show a moderate, but definite, reaction. PAS method. ×600.

FIG. 10.—Chloroform–methanol extracted peripheral nerve, longitudinal section. The segments of neurokeratin networks are no longer evident but the endoneurium (*E*) is deeply stained. Two constrictions in the endoneurium indicate the sites of nodes of Ranvier (*N*). PAS method. ×600.

FIG. 11.—Denatured proteolipide protein film. The coagulated protein has taken the form of a reticulum which resembles somewhat the appearance of the neurokeratin network, and like the latter, is intensely stained. WEIL stain. ×600.

FIG. 12.—Denatured proteolipide protein film. The coagulated protein shows a coloration which is only moderate as compared with that in Fig. 11. PAS method. ×600.

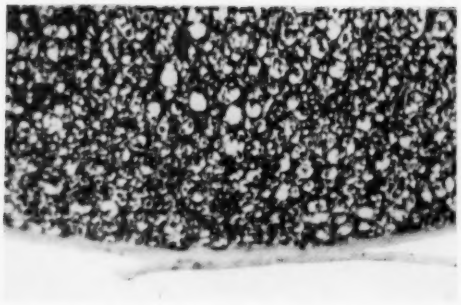

FIG. 13. White matter of spinal cord, transverse section, formalin-fixed. Note the intense black staining of the neurokeratin network. WEIL stain. ×70.

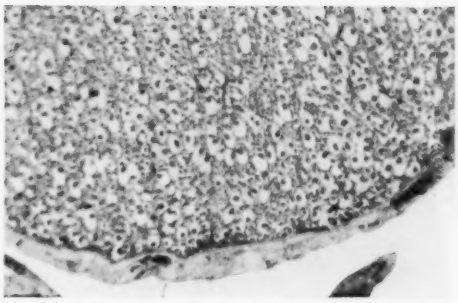

FIG. 14. White matter of spinal cord, transverse section, soaked in chloroform–methanol prior to fixation. The black staining of neurokeratin is abolished and in its place, the axons stain brown. WEIL stain. ×70.

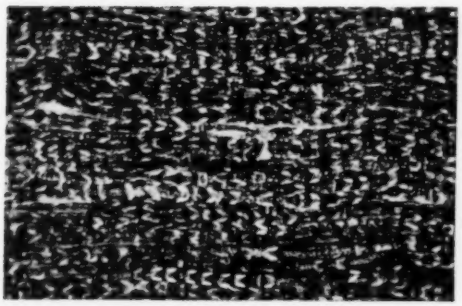

FIG. 15. Peripheral nerve, longitudinal section, formalin-fixed. The neurokeratin segments are stained black. WEIL stain. ×70.

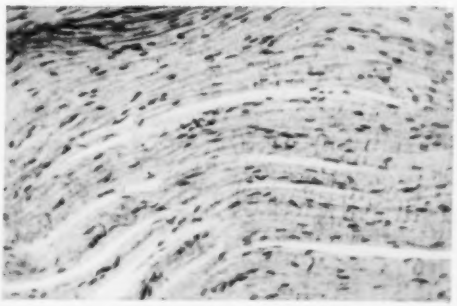

FIG. 16. Peripheral nerve, longitudinal section, soaked in chloroform–methanol before fixation. The black staining of neurokeratin segments is absent. Nuclei of Schwann cells are conspicuously stained. WEIL stain. ×70.

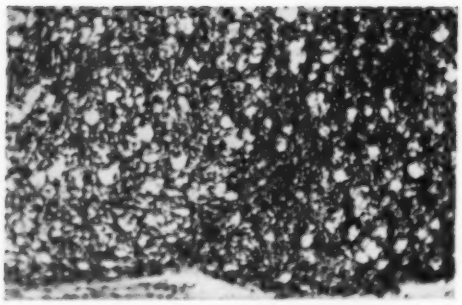

FIG. 17. White matter of spinal cord, transverse section, formalin-fixed. The neurokeratin is stained blue-violet. KLÜVER-BARRERA stain. ×70.

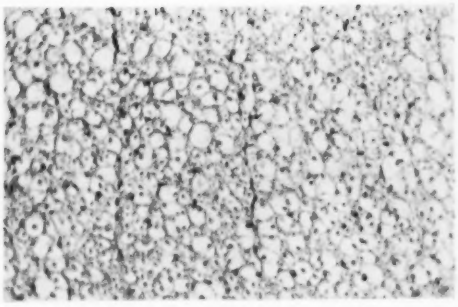

FIG. 18. White matter of spinal cord, transverse section, soaked in chloroform-methanol prior to fixation. The blue-violet staining of neurokeratin is abolished. Axons are stained a clear blue. KLÜVER-BARRERA stain. ×70.

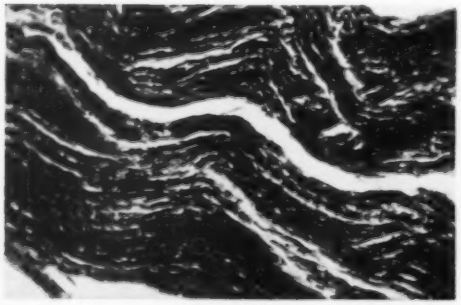

FIG. 19. Peripheral nerve, longitudinal section, formalin-fixed. Neurokeratin segments are stained an intense violet. KLÜVER-BARRERA stain. ×70.

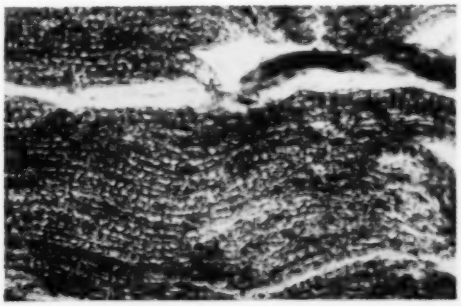

FIG. 20. Peripheral nerve, longitudinal section, soaked in chloroform-methanol before fixation. The violet-stained neurokeratin segments are not present. KLÜVER-BARRERA stain. ×70.

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as in white matter. This is attributed to the staining of Schwann and endoneurial sheaths present in peripheral nerve (Figs. 6, 8, 16 and 20).

Proteolipid protein films

Denatured proteolipid protein films stained strongly with the WEIL, KLÜVER–BARERRA, thionin and fast green methods. The periodic acid–Schiff method gave a moderate, but definite reaction. The direct Schiff reaction and acetylation blockade gave a trace reaction and no reaction respectively. Microscopically, much of the protein in the film had the appearance of a vacuolated reticulum not unlike the neurokeratin network of myelin. The staining of this artificially prepared reticulum by the aforementioned methods resembled closely, both in colour and intensity, the staining of neurokeratin in formalin-fixed tissue sections (Figs. 11 and 12).

DISCUSSION

The staining of 'myelin' in ordinary paraffin-imbedded tissue sections by the WEIL and the KLÜVER methods is actually a staining of the myelin protein, i.e. the neurokeratin network, for the myelin lipids are dissolved by solvents during the processes of infiltration and imbedding. The myelin sheath can be protected from lipid solvents and preserved through paraffin imbedding by prior chromation or osmification. The avoidance of lipid solvents by the use of fresh or fixed frozen sections accomplishes the same end. Yet even when myelin lipids are preserved, the neurokeratin network may be stained more heavily than the lipids by methods presumably specific for lipid components. According to WISLOCKI and SINGER (1950), the Sudan black B stain for lipids, Baker's acid haematin stain for phospholipids and the Pal–Weigert stain for myelin all stain the neurokeratin network more heavily than the myelin lipids in frozen sections.

A loss of myelin staining would be expected to accompany the removal of the neuroketatin reticulum. This loss of staining and removal of the neurokeratin network has been accomplished by soaking neural tissues in chloroform–methanol before fixation. This solvent removes proteolipids and strandin as well as lipids (FOLCH, ASCOLI, LEES, MEATH and LEBARON, 1951). If tissues are fixed initially, the protein moiety of proteolipid is denatured and resists extraction by lipid solvents, although the lipid component is readily extracted. Strandin, which appears to be bound to protein (FOLCH, ARSOVE and MEATH, 1951), may remain in the tissue after such treatment. However, strandin probably does not contribute to the neurokeratin reticulum of fixed tissue as it is present only in trace amounts in white matter. The extraction of the precursor of the neurokeratin network from neural tissue by chloroform–methanol and the consequent loss of 'myelin' staining suggest that the neurokeratin network of myelin sheaths consists principally, if not entirely, of proteolipid protein. This inference is corroborated by the finding that proteolipid protein films are stained intensely by the WEIL and KLÜVER–BARRERA methods. KLÜVER and BARRERA (1954) reported that soaking fresh brain in a variety of lipid solvents prior to fixation abolished the staining of myelin sheaths by their methods. It appears likely that the failure of myelin staining was caused by extraction of proteolipid protein. However, the condition of the neurokeratin reticulum following such treatment was not described.

The difference between myelin sheaths of the central and peripheral nervous

system merits consideration. Central myelin is found in the proximal or glial segments of cranial and spinal nerves and throughout the optic nerves as well as in the central nervous system. The glia, principally interfascicular oligoglia, comprise the cellular sheath of central myelin. The Schwann cells, form a sheath, neurilemma, for peripheral myelin. In addition the neurilemma is invested by connective tissue elements which comprise the endoneurium of peripheral nerve fibres. An endoneurium is lacking where central myelin is present. The cellular and mesodermal investments of peripheral myelin sheaths have been the object of extensive study (reviewed by NAGEOTTE, 1932).

The differences in the neurokeratin network of central and peripheral myelin seem to have little received notice. TARLOV (1937) and RASMUSSEN (1946) both noted the heavier staining of peripheral myelin in the neurilemmal portion of the glia–Schwann cell junction of cranial and spinal nerve roots which is described in this paper. The lamellae of central and peripheral myelin appear similar in electron micrographs. However, a subtle ultrastructural difference between central and peripheral myelin has been detected by electron microscopy (FERNÁNDEZ-MORÁN, 1957) and X-ray diffraction studies (FINEAN, 1957). There is, perhaps, a relationship between this difference and the difference in the neurokeratin network. The course of Wallerian degeneration is different in central and peripheral myelin (JAKOB, 1912; RASMUSSEN, 1946). However, this difference is probably one of cellular response to and removal of products of degenerating myelin, rather than a difference in the manner by which central and peripheral myelin degenerate following axonal injury (NOBACK and REILLY, 1956). The biochemical alterations which accompany Wallerian degeneration in central and peripheral myelin (MCCAMAN and ROBINS, 1958) can be explained in the same way.

Although differing in form, the neurokeratin networks of central and peripheral myelin are similar with respect to staining properties and solubility in chloroform–methanol. For reasons already cited, this network is considered to be denatured proteolipid protein. In contrast to these results, FINEAN, HAWTHORNE and PATTERSON (1956) obtained only negligible amounts of proteolipid protein from peripheral myelin (sciatic nerve) compared with central myelin (optic nerve). Since chloroform–methanol appears to remove proteolipid from peripheral myelin as well as from central myelin, it may be that the method of FOLCH and LEES (1951), which FINEAN and his associates employed, does not effect a quantitative recovery of proteolipid from the chloroform–methanol extract of peripheral nerve. If this is correct, it would indicate that proteolipid in central and peripheral myelin are physicochemically different. The findings of FINEAN *et al.* (1956) that proteolipid protein from peripheral nerve has half the inositol and phosphorus that proteolipid protein from optic nerve contains is consistent with this hypothesis. This problem deserves further study.

The neurokeratin network in formalin-fixed tissue sections behaves as do other tissue proteins with respect to staining with acidic and basic dyes, i.e. it is amphoteric. At low pH the ionization of basic groups, principally amino radicals, is favoured and leads to the binding of acidic (anionic) dyes; at pH above the isoelectric point of protein, the ionization of acidic groups, principally carboxyl radicals, is promoted with resultant binding of basic (cationic) dyes. Such dye binding appears to be brought about through the formation of salt linkages (SINGER, 1952). Although dye binding by the neurokeratin network was not investigated over the full range of pH

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with basic and acidic dyes, it appeared that this component bound thionin, a basic dye, more strongly than fast green, an acidic dye. The relative basophilia of the neurokeratin network is probably caused by the fixative employed, for formalin is known to increase the staining of tissue proteins by basic dyes through binding of amino groups (SINGER, 1952). The basophilia cannot be attributed to the amino acid composition of proteolipid protein, since dicarboxylic amino acids are present in relatively small amounts in this substance (FOLCH and LEES, 1951). This question requires further investigation.

Several workers (WISLOCKI and SINGER, 1950; HESS, 1953) have reported that the neurokeratin network is not stained by the PAS method. NOBACK and MONTAGNA (1952), on the other hand, obtained a positive reaction with this stain but attributed it to the presence of lipid in the neurokeratin reticulum. We found that the neurokeratin network and proteolipid protein films both gave a positive PAS reaction. This finding together with the marked suppression of the reaction brought about by omitting the periodic acid oxidation and by acetylation blockade prior to PAS staining suggest the presence of vicinal OH groups or OH and NH_2 groups in proteolipid protein (LILLIE, 1954). Inositol, a cyclic polyalcohol, occurs in proteolipid protein (FINEAN, HAWTHORNE and PATTERSON, 1956) and may be responsible for the PAS reaction shown by this substance.

The staining of axons in chloroform–methanol extracted neural tissue by all five staining methods is remarkable. The neurokeratin network has a strong affinity for these stains and, when present, not only obscures the axons but also interferes with the staining of axoplasm. KLÜVER and BARRERA (1954) reported a staining of axis cylinders by their method along with a loss of myelin staining when blocks of brain were soaked in lipid solvents before fixation. The success enjoyed by CAJAL and others in staining axis cylinders in the neuraxis of foetal and neonatal animals by silver methods is attributable, in part at least, to the absence of myelin proteolipid. Prior extraction of proteolipid protein from spinal cord and peripheral nerve improves the staining of axones by Bodian's silver method (KOENIG and RICH, 1958). However, synaptic terminals seem to be dissolved by chloroform–methanol. The effect of removal of proteolipid protein on the staining of other structural elements in the nervous system is under investigation.

SUMMARY

The neurokeratin network of myelin sheaths in white matter of spinal cord and in peripheral nerve was the object of histochemical analysis. The neurokeratin network of myelin sheaths in formalin-fixed, lipid-extracted tissues was intensely stained by the WEIL and KLÜVER–BARRERA methods for myelin and by thionin at pH 7·4. Fast green at pH 0·9 and the periodic acid–Schiff technique also stained the neurokeratin reticulum, although less intensely. Morphological differences in the neurokeratin network of central and peripheral myelin were described.

When neural tissues were soaked in chloroform–methanol (2 : 1 v/v) prior to fixation in formalin, 'myelin' staining was abolished. The neurokeratin network in white matter of spinal cord and in peripheral nerve remained unstained by all five staining methods; in its place was empty space. Axons, both large and small, were deeply stained by the WEIL, KLÜVER–BARRERA, thionin and fast green methods; indeed, the axons appeared as if they had been selectively stained.

The abolition of the neurokeratin network and 'myelin' staining brought about by the soaking of neural tissues in chloroform–methanol suggested that the neurokeratin network is derived from proteolipid protein since the latter is soluble in this solvent. This inference was corroborated by the finding that films of denatured proteolipid protein were stained in the same manner as the neurokeratin network. It was suggested that the preliminary removal of proteolipid protein might facilitate the staining of special neural structures.

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HISTOCHEMICAL INVESTIGATIONS ON SUCCINIC DEHYDROGENASE IN THE CENTRAL NERVOUS SYSTEM—I

THE POSTNATAL DEVELOPMENT OF RAT BRAIN

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THE development of the brain is reflected in an increase of many substances and enzymes. One of these, succinic dehydrogenase, which is dealt with in this paper, has been shown to increase sharply in the cortex of the pig and guinea pig coinciding with periods of morphological differentiation (FLEXNER and FLEXNER, 1946). In the rat brain, succinic dehydrogenase activity (SDA) was found to increase rapidly after the sixth day *post partum* (POTTER, SCHNEIDER and LIEBL, 1945).

The present paper reports changes in the histochemical distribution of succinic dehydrogenase activity (SDA) in the grey matter of the rat brain during postnatal development. By histochemical techniques more detailed information may be obtained on the behaviour of individual nuclei than by biochemical techniques. The present article will be followed by one providing a detailed description of the distribution of SDA in the brain of the adult guinea pig. Since the distribution of SDA in the brains of adult guinea pigs and adult rats is very similar, this subsequent article may be used as a reference on the distribution of SDA in the adult rat brain. The actual distribution of SDA in the adult rat brain has been described previously by SHIMIZU and MORIKAWA (1957) employing a somewhat different technique.

MATERIALS AND METHODS

Newborn albino rats were sacrificed by decapitation on the 1, 2, 3, 5, 7, 9, 10, 12, 14, 16, 18, and 20th day *post partum*, and the brain was removed from the cranial vault as quickly as possible. Complete series of unfixed frozen sections were cut. The preparation of such unfixed sections from the very soft brain of newborn rats was difficult and required sections 80 μ in thickness. This thickness was also used in sectioning the brains of elder animals to afford better comparisons. A tissue temperature of -1–$3°$C had to be maintained during the cutting to insure the later demonstration of SDA in small and delicate structures. It is imperative to cut the specimens within this range of temperature which may be controlled by a thermometer built into the microtome holder. This modified technique has been described in detail in an earlier publication (FRIEDE, 1958).

The sections are transferred from the knife directly into the incubation fluid which contains equal parts of a 1% solution of 4:4-bis(3:5-diphenyl-2-tetrazolium-biphenyl) chloride (Tetrazolpurpur, Bayer), a 0·25% solution of sodium succinate, and a 0·1 M-phosphate buffer of pH 7·8. The sections are incubated for 2 hr at 38° in tightly closed weighing bottles and then fixed by pouring in formalin. No counterstain is used and the sections are mounted with glycerin gelatine.

The SDA results in the deposition of dark violet diformazan granules. The density of the granules was used as an index of the enzyme activity. Since the density of the reaction was also a function of

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the incubation time, temperature, etc., the conditions of the reaction were standardized carefully to obtain comparable results. Besides the granular reaction, a yellow or orange diffusely stained background was produced, involving a chemically different reaction (BURTNER, BAHN and LONGLEY, 1957). This diffuse staining of the background was exceedingly weak compared to the granular reaction and thus is termed herein a negative reaction. Heating of the sections before incubation resulted in no reaction; blocking of SDA by addition of adequate concentrations of a malonate or selenite inhibited the reaction, thus proving its specificity.

The pattern by which SDA increases during the postnatal period is given in tabular form (Tables 1 to 3), and the text is limited to a description of general characteristics or unusual behaviour of individual nuclei. The intensity of SDA may be divided conveniently into three grades. A weak, grade 1, reaction is demonstrated by a few, scattered formazan granules; a mild, grade 2, reaction is shown by a moderate amount of granules; and, a strong, grade 3 reaction is marked by the first appearance of dense formazan granules. This last grade of reaction, however, is not meant to imply that SDA may not further increase during the following days, but any such further increase is not indicated in the tables because of the subjective difficulties in determining quantitatively an increase in an already so strong recation. Also, histochemical techniques are always more suitable for detecting local changes in a pattern of distribution than for detecting general changes occurring throughout an already established pattern.

The classification used above is appropriate, for most of the nuclei show strong SDA in the adult animal. A small number of nuclei, however, continue to show weak SDA or even a decrease in SDA in the adult animal, and the extraordinary behaviour of these nuclei during development must be emphasized, since the tabular classification cannot sufficiently express the actual changes.

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RESULTS

In most of the nuclei the basic changes occurring during development are simple. Sometimes a very dense accumulation of SDA is found initially, reflecting the early compactness of a nucleus and obscuring finer constituent details as cells and neuropil. Generally, though, cell bodies are distinguishable in the early stages on a negative or almost negative background. The SDA in the neuropil increases later. In some nuclei, as the medial vestibular nucleus or the habenula the SDA in both the cell bodies and the neuropil increases simultaneously, and thus outlining of the cell bodies is indistinct at any stage. The cerebral and cerebellar cortex show a complicated development passing through several stages of enzyme distribution.

Pia mater and arachnoid membrane. Pia mater and arachnoid membrane could not be separated clearly enough in the early stages and are referred to herein just as 'meninges'. Within the first few days, SDA in the meninges frequently exceeds that in the brain tissue. Especially a thickening of the meninges in the angle between the pons and mammillary bodies, covering the ventral surface of the cerebral peduncles, consistently contained strong SDA. Nowhere else in the brain was such strong SDA found on the first day. Until about the fifth day, the SDA in this area exceeds that in the adjacent brain nuclei, such as the substantia nigra, red nucleus, etc. Such strong SDA reflects the very active metabolism of this structure in the early stages of development suggesting an unknown functional significance. Other portions of the meninges also may exhibit a patchy distribution of mild or strong SDA, but nowhere was there as constant and as strong an activity on the first day.

Ependyma and choroid plexus. On the first day, only the ependyma of the ventral wall of the cerebral aqueduct shows SDA. The epithelium of the choroid plexus shows weak SDA. A rapid increase of activity occurs within the following days both in the ependyma and the choroid plexus. Strong SDA in these structures is found on the third day, but the cells of the median sulcus of the fourth ventricle remain SDA negative until about the ninth day. The ependyma of the central canal of the spinal

cord is negative until the twentieth day, the end of the developmental period studied, but exhibits strong SDA in adult animals as does the remainder of the ependyma and the epithelium of the choroid plexus. Only the ependyma of the recessus infundibuli and of the subcommissural organ permanently lack SDA.

White matter. The white matter generally shows a negative reaction throughout development. During myelination, however, abundant SDA-positive cells, obviously glia cells, are seen in the white matter. They disappear after myelination. This specific finding during myelination will not be dealt with in this article, since for a full understanding it is felt that more elaborate investigations with various techniques are required.

Hindbrain. Three types of nuclei are discernable in the hindbrain by the period of their increase in SDA: (a) nuclei with strong SDA at birth, (b) nuclei gaining strong SDA within the first 3 days, and (c) nuclei with a delayed increase of SDA.

Only a small number of nuclei display strong SDA within hours after birth: the anterior grey column of the spinal cord, the hypoglossal nucleus, the nucleus of

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TABLE 1.—HINDBRAIN*

	Days *post partum*											
	1	2	3	5	7	9	10	12	14	16	18	20
Anterior column of spinal cord	3	3	3	3	3	3	3	3	3	3	3	3
Nucleus hypoglossus	3	3	3	3	3	3	3	3	3	3	3	3
Reticular substance of medulla oblongata	–	2	3	3	3	3	3	3	3	3	3	3
Nucleus of spinal tract of n. trigeminus	–	–	–	1	1	2	3	3	3	3	3	3
Nucleus gracilis	–	–	–	–	–	2	3	3	3	3	3	3
Medial cuneate nucleus	–	–	–	–	–	2	3	3	3	3	3	3
Lateral cuneate nucleus	–	2	3	3	3	3	3	3	3	3	3	3
Dorsal motor nucleus of vagus	1	2	2	3	3	3	3	3	3	3	3	3
Nucleus of tractus solitarii	–	2	2	3	3	3	3	3	3	3	3	3
Glial tissue of the obex	–	–	–	–	–	2	2	3	3	3	3	3
Nucleus ambiguus	3	3	3	3	3	3	3	3	3	3	3	3
Inferior olivary nucleus	–	–	–	–	–	2	2	3	3	3	3	3
Nucleus prepositus hypoglossi	–	3	3	3	3	3	3	3	3	3	3	3
Nucleus funiculi lateralis (Winkler, Potter)	–	–	1	2	3	3	3	3	3	3	3	3
Spinal vestibular nucleus	–	3	3	3	3	3	3	3	3	3	3	3
Medial vestibular nucleus	–	3	3	3	3	3	3	3	3	3	3	3
Lateral vestibular nucleus (Deiters)	–	3	3	3	3	3	3	3	3	3	3	3
Superior vestibular nucleus (Bechterew)	–	3	3	3	3	3	3	3	3	3	3	3
Acoustic tubercle	–	3	3	3	3	3	3	3	3	3	3	3
Cochlear nucleus	–	3	3	3	3	3	3	3	3	3	3	3
Nucleus facialis	2	3	3	3	3	3	3	3	3	3	3	3
Nucleus of trapezoid body	–	3	3	3	3	3	3	3	3	3	3	3
Superior olivary nucleus	–	–	1	1	2	2	3	3	3	3	3	3
Superior parolivary nucleus	–	3	3	3	3	3	3	3	3	3	3	3
Nucleus abducens	–	3	3	3	3	3	3	3	3	3	3	3
Reticular substance of pons	–	3	3	3	3	3	3	3	3	3	3	3
Motor nucleus of trigeminal nerve	2	3	3	3	3	3	3	3	3	3	3	3
Main sensory nucleus of trigeminal nerve	–	–	1	2	2	3	3	3	3	3	3	3

* Definition of the grades of enzyme activity in the text.

Roller, and the nucleus ambiguus caudalis. A weak granular reaction is found in the facial and trigeminal motor nuclei. No SDA is found in the other nuclei or in the white matter (Fig. 1).

An additional number of nuclei exhibit SDA on the second day: the reticular formation, the lateral cuneate nucleus, nucleus prepositus hypoglossi, all the vestibular nuclei, the acoustic tubercle, the cochlear nucleus, the nucleus of the trapezoid body, and the facial, abducens, and trigeminal motor nuclei. In the acoustic tubercle, the SDA in the superficial molecular layer appears later than in the deeper layers. The nucleus ambiguus caudalis and the reticular formation show SDA in large dendrites over vast distances. In the medial vestibular nucleus SDA appears simultaneously in the cell bodies and the neuropil.

In the following days, SDA increases in all these nuclei, but some other nuclei continue to show very weak or no SDA. Such nuclei include the medial cuneate nucleus and nucleus gracilis, the nucleus of the spinal trigeminal tract as well as the principal sensory trigeminal nucleus and the superior olivary nucleus. The inferior olivary nucleus is about the last nucleus of the hindbrain to gain strong SDA, which increases in this nucleus on about the eleventh day.

The dorsal vagal nucleus shows weak SDA in adult animals but gains this weak SDA relatively early. After the first week, the SDA in this nucleus somewhat exceeds the amount found in adult animals. A similar but more pronounced behaviour is found in the nucleus coeruleus and will be discussed later in detail.

The permanent distribution of SDA in the hindbrain has developed at about the twelfth to fourteenth day. The total amount of SDA in the third week may possibly exceed the normal level in adult animals, but only biochemical techniques are capable of establishing the presence of such a temporary surplus.

Cerebellum. Apart from the cerebral cortex, the cerebellar cortex has the most complicated pattern of postnatal development. Its description in this article is limited to an abstract, since a detailed description of the development of the rat's cerebellum was published in detail (FRIEDE, 1957).

The cerebellar cortex shows no SDA on the first day, but on the second day strong SDA is already visible in the Purkinje cells bodies. Short straight dendrites of the Purkinje cells exhibit SDA on the ninth day, and branching of these dendrites is seen on the twelfth day. A diffusely arranged SDA of the neuropil between the dendrites appears at the fourteenth day and is limited to the deeper portions of the molecular layer. Within the next few days the SDA of the neuropil increases towards the surface of the molecular layer, finally reaching it by the sixteenth to twentieth day. The granular layer of the cerebellar cortex shows the first appearance of SDA at the ninth day and has gained the adult appearance by the fifteenth to sixteenth day. The embryonic superficial layer shows weak SDA throughout development until it disappears.

The SDA in the cerebellar nuclei develops between the second and twelfth day, showing early in the cell bodies and later in the neuropil. The adult distribution of SDA is present in the cerebellar nuclei about 8 days prior to that in the cerebellar cortex (Fig. 2).

Midbrain. The adult distribution of SDA is found in the midbrain later than in the hindbrain. On the first day SDA is found in the mesencephalic trigeminal nucleus, the oculomotor and trochlear nuclei, the magnocellular part of the red nucleus, and

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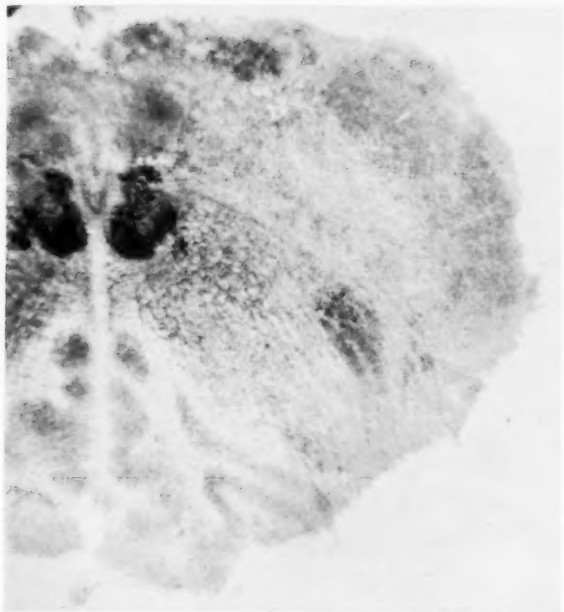

FIG. 1.—Medulla oblongata, second day. Enzyme activity is found almost exclusively in the hypoglossal nucleus and the nucleus ambiguus. ×26. (This picture should be compared with Plate III of the atlas of distribution of SDA in the medulla oblongata of the guinea pig; following article).

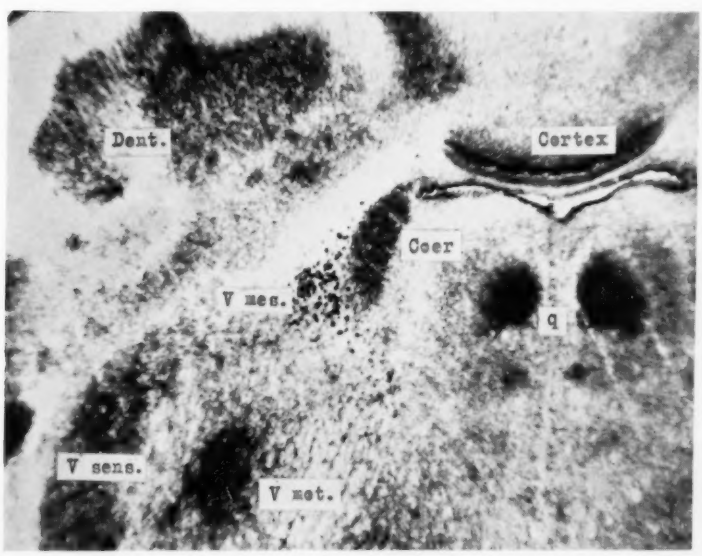

FIG. 2.—Pons, fifth day. Enzyme activity is found in the main sensory (V. sens.) and motor (V. mot.) trigeminal nucleus, the mesencephalic trigeminal nucleus (V. mes.), the nucleus dentatus (Dent.), the cerebellar cortex (Cortex), the area *q* of Meessen and Olszewski (*q*) and the nucleus coeruleus (coer.). In the adult rat the enzyme activity is further increased in most of the nuclei but the nucleus coeruleus then almost completely lacks SDA. ×26.

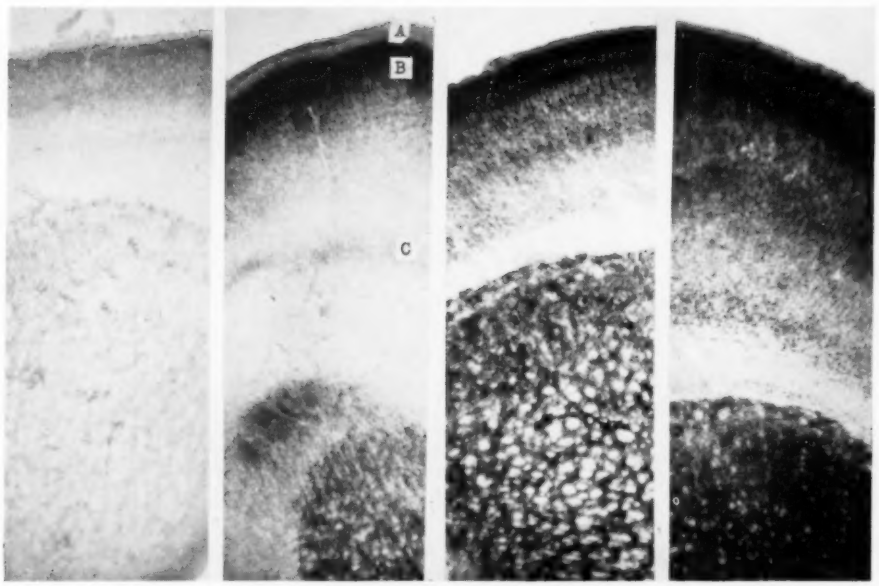

FIG. 3.—Cerebral cortex and putamen, first, second, fifth and tenth day. Gradual establishment of the laminar arrangement of the cortex and cell isles of strong SDA in the putamen. ×26.

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the lateral portion of the substantia nigra. The SDA in the cells of the mesencephalic trigeminal nucleus shows its permanent distribution from the first day on. These cells react intensely without any corresponding reaction in the neuropil. Since the neuropil never develops strong SDA, both this typical pattern and its early establishment are rather unique in the entire brain.

TABLE 2.—MIDBRAIN*

	Days *post partum*											
	1	2	3	5	7	9	10	12	14	16	18	20
Mesencephalic nucleus of trigeminal nerve	3	3	3	3	3	3	3	3	3	3	3	3
Regio Q of Meessen and Olszewski	–	2	3	3	3	3	3	3	3	3	3	3
Nucleus coeruleus	–	–	1	3	3	3	3	3	3	3	3	3
Nucleus parabrachialis (brachium conjunctivum)	–	1	1	1	2	3	3	3	3	3	3	3
Nuclei pontis	–	1	2	3	3	3	3	3	3	3	3	3
Nuclei lemnisci lateralis	–	1	1	3	3	3	3	3	3	3	3	3
Central grey stratum of the aqueduct	–	–	–	1	2	3	3	3	3	3	3	3
Nucleus of inferior colliculus	–	–	1	1	1	2	3	3	3	3	3	3
Superficial layer of superior colliculus	–	–	–	1	1	1	1	1	2	3	3	3
Deep layers of superior colliculus	–	–	–	1	1	2	3	3	3	3	3	3
Nucleus dorsalis tegmenti	–	–	2	2	3	3	3	3	3	3	3	3
Nucleus trochlearis	3	3	3	3	3	3	3	3	3	3	3	3
Nucleus oculomotorius	3	3	3	3	3	3	3	3	3	3	3	3
Nucleus of Edinger Westphal	–	–	1	2	3	3	3	3	3	3	3	3
Nucleus of Darkschewitsch	–	1	1	3	3	3	3	3	3	3	3	3
Red nucleus, magnocellular portion	3	3	3	3	3	3	3	3	3	3	3	3
Red nucleus, parvocellular portion	–	–	1	2	3	3	3	3	3	3	3	3
Substantia nigra, pars compacta	–	–	–	–	1	2	2	3	3	3	3	3
Substantia nigra, pars reticularis	1	2	2	3	3	3	3	3	3	3	3	3
Substantia nigra, pars lateralis	3	3	3	3	3	3	3	3	3	3	3	3
Interpeduncular ganglion	–	1	1	2	2	2	3	3	3	3	3	3

* Definition of the grades of enzyme activity in the text.

Most intriguing is the behaviour of the nucleus coeruleus and the pars compacta of the substantia nigra; both nuclei show very weak SDA in the adult animal. The cells of the nucleus coeruleus almost completely lack SDA. These two nuclei, however, show mild to increasingly strong SDA after the first 7 days, definitely exceeding the adult level during this period of development. A similar behaviour was noted in the dorsal vagal nucleus. Most interestingly, such outstanding activity was coincident with the presence in adult man and some mammals of melanin within the nuclei. Thus, the question rises whether the appearance of the melanin could be related to a temporary hyperactivity during development (Fig. 2). The nucleus of Edinger Westphal exhibits weak SDA throughout life.

Remarkable also is the delayed increase of SDA in the superficial layer of the superior quadrigeminal body on the sixteenth day. SDA is found in the deeper cellular layers on the tenth day. The late appearance of SDA in all the so-called molecular layers (cerebellum, acoustic tuberlce, anterior quadrigeminal body, cerebral cortex) seems a rather typical feature of such structures.

The identification of the nuclei of the midbrain is difficult in the early stages of development. The nucleus mesencephalicus profundus (nucleus of Gudden), for instance, seems to show strong SDA on the first day, but its identity could not be established beyond doubt because of the lack of other landmarks.

TABLE 3.—DIENCEPHALON AND BASAL GANGLIA*

	Days *post partum*											
	1	2	3	5	7	9	10	12	14	16	18	20
Habenular nuclei	2	3	3	3	3	3	3	3	3	3	3	3
Anterior thalamic nuclei	1	3	3	3	3	3	3	3	3	3	3	3
Dorsolateral thalamic nuclei	2	3	3	3	3	3	3	3	3	3	3	3
Ventrolateral thalamic nuclei	1	2	2	3	3	3	3	3	3	3	3	3
Medial thalamic nuclei	1	1	2	3	3	3	3	3	3	3	3	3
Posterior thalamic nuclei	–	–	–	1	1	1	2	3	3	3	3	3
Lateral geniculate body	–	–	–	2	2	3	3	3	3	3	3	3
Medial geniculate body	–	–	–	2	2	3	3	3	3	3	3	3
Reticular nucleus of thalamus	–	–	–	3	3	3	3	3	3	3	3	3
Subthalamic nucleus	2	3	3	3	3	3	3	3	3	3	3	3
Hypothalamus (tuber cinereum)	1	3	3	3	3	3	3	3	3	3	3	3
Medial nucleus of mammillary bodies	–	–	–	–	–	–	–	2	3	3	3	3
Lateral nucleus of mammillary bodies	2	3	3	3	3	3	3	3	3	3	3	3
Putamen	–	2	2	2	3	3	3	3	3	3	3	3
Pallidum	–	2	2	2	3	3	3	3	3	3	3	3
Amygdaloid nucleus	–	–	3	3	3	3	3	3	3	3	3	3

* The gradations in this table provide a very general and approximate measure, since the actual changes in the enzyme distribution are complicated and beyond the scale of a simple gradation.

Diencephalon and basal ganglia. Some of these nuclei, especially the putamen and the thalamus, are characterized by a relatively early appearance of SDA but a delayed development of the adult pattern. The rather slow or complicated development of these regions is shared by the cerebellar and cerebral cortex in contrast to the simple pattern of development found in most of the nuclei of the hindbrain.

An early increase of SDA is observed in the thalamus in the anterior nuclei and in the habenula, the latter exhibiting SDA in the neuropil on the first day. The ventrolateral thalamic nucleus, on the other hand, shows SDA in cell bodies from the second to twelfth day, and the SDA in the neuropil appears after the twelfth day. A similarly delayed increase of SDA in the neuropil is found also in the dorsolateral and medial thalamic nuclei, although not being as pronounced.

A slow but continuous increase of SDA was found in the putamen, extending over a period of 12 days. During this period a patchy pattern appears in the putamen by the formation of small cell isles in which SDA is stronger in both neuropil and cell bodies than in the remaining portions of the nucleus. The patchy pattern is pronounced in the first days and disappears gradually until the twelfth day; it is typical for the putamen and unique in the entire brain. After the fourteenth day a diffuse arrangement of strong SDA in the neuropil is found throughout the putamen. The pallidum also shows a delayed development, but not so slow as that of the putamen.

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SDA in the hypothalamus and the subthalamic nucleus appears already on the second day in contrast to the protracted development of the above mentioned nuclei. Also remarkable are the different behaviours of the lateral and medial subnuclei of the mammillary body, the former showing SDA on the second day, but the latter not until the fourteenth day.

Cerebral cortex. The cerebral cortex shows a continual increase of SDA during the first 3 weeks, and the distribution of SDA changes several times. Since the same changes are not found in all cortical areas, the following description is concerned only with the sensory regions which show a strongly SDA-positive neuropil in the Brodmann's third layer in adult animals.

In the early stages of development three main layers are discernable in the neocortex, two of which are present on the first day: a thin superficial layer *A* without SDA, and a thicker layer *B* of diffusely arranged SDA which grades off towards the white matter. On the second day a thin layer *C* of larger cells with mild SDA appears in the deepest portion of the cortex, isolated from the layer *B* by an SDA-negative zone.

Changes during the following days occur in the middle, thick layer *B*: It differentiates into several laminae, the SDA spreading through layer *A* towards the surface of the cortex and also connecting with the deeper cellular layer *C*. SDA in the upper lamina of the layer *B* increases progressively up to the fifth day. This lamina then moves towards the surface of the cortex, which it reaches on the fourteenth day. At this time the lamina occupies the superficial portion of the cytoarchitectonic molecular layer. The lower portion of the layer *B* differentiates into several laminae during the fifth to fourteenth day. Below the above mentioned superficial lamina, in the deeper portions of the molecular layer, the diffuse SDA grades off somewhat, but increases again in the dense neuropil of the cytoarchitectonic second and third layers. At the level of the cytoarchitectonic fourth and fifth layers SDA is predominantly in the cell bodies. That the deep layer *C* is identical with the sixth cortical layer does not seem quite certain. This deep layer *C* is recognizable as a separate layer up to the fourteenth day. By the sixteenth day this layer is mingled with the adjacent upper layers and thus is no longer discernable as a separate entity, and the adult distribution of SDA is reached.

The tetrazolium technique permits sufficient differentiation between cortical areas, but a detailed description of the areas is beyond the scope of this article. Differences in the distribution of SDA between areas of the neocortex were observed from the seventh day on, while differences between the isocortex and the allocortex were found as early as the second day. It is remarkable that the temporal cortex eventually shows lower SDA than the remainder of the adult neocortex.

The cell layer of the fascia dentata and the hippocampus exhibit strong SDA on the first day and mild SDA of the plexiform layers during the following days.

The periventricular, undifferentiated, embryonic tissue shows weak SDA on the second day. A slight increase of SDA occurs thereafter, but at no time is a strong SDA found. Scattered cells with a mild SDA are found in the white matter near the periventricular tissue on the third day. They are obviously identical with migrating embryonal neuroblasts. At this stage the walls of proliferating blood vessels in the white matter also show mild reaction which is not present in adult animals.

DISCUSSION

The demonstration of succinic dehydrogenase activity (SDA) in tissue implies a functional tricarboxylic–dicarboxylic acid cycle (Krebs cycle). Since succinic dehydrogenase is localized in the mitochondria of the cell in association with cytochrome oxidase, the demonstration of succinic dehydrogenase implies the presence of a functional cytochrome respiratory system in the tissue. The concentration of succinic dehydrogenase in tissue may be assumed to be a parameter for its oxidative metabolism and general metabolic activity.

The postnatal increase of succinic dehydrogenase in the rat brain found in the present histochemical investigation agrees with the increase of the enzyme after the sixth day found in earlier biochemical analyses (POTTER, SCHNEIDER and LIEBL, 1945). The histochemical technique, therefore, has sufficient quantitative accuracy. The increase in succinic dehydrogenase reflects the increase of oxygen consumption during development of the brain found in rats (HIMWICH, SYKOWSKI and FAZEKAS, 1941, TYLER and VAN HARREVELD, 1942), pigs (FLEXNER, FLEXNER and STRAUSS, 1941), and dogs (HIMWICH and FAZEKAS, 1941). The undeveloped brain seems not nearly as dependent on oxygen as the adult, for newborn animals will survive anoxia far longer than adults (FAZEKAS, ALEXANDER and HIMWICH, 1941) and they are less sensitive to arrest of circulation (KABAT, 1940). Malanates, which inhibit respiration in the adult rat brain by inhibiting succinic dehydrogenase, have little effect on newborn rat brains, but an increased sensitivity to them developes very quickly within 10 days after birth (TYLER, 1942). A malonate, on the other hand, is also used to control the specificity of the histochemical tetrazolium reaction. Thus, the same mechanism of inhibition of succinic dehydrogenase produces the inhibition of respiration and can also be visualized by the prevention of the staining reaction.

In reference to a metabolic differentiation of areas of the brain, the increase in oxygen consumption in newborn animals has been shown by biochemical technique to occur rostrally from the medulla to the caudate nucleus (HIMWICH and FAZEKAS, 1941; TYLER and VAN HARREVELD, 1942). The presented histochemical changes are in agreement with these results in showing the establishment of the adult distribution of SDA earlier in the caudal than in the rostral portions of the brain. However, much more detailed information on the pattern of development is obtainable by the histochemical technique.

The concept of a caudo–cranial maturation, though, does not give sufficient credit to the exceptional behaviour of particular nuclei for most nuclei of the hindbrain show strong SDA by the third day, but some of them have a delayed development. On the other hand, some of the diencephalic nuclei show very early a strong activity, but they are outnumbered by those having a delayed increase of activity. Therefore, the onset of development seems a rather specific characteristic of any nucleus and not of the general area in which it is located. In the hindbrain, for example, the motor nuclei seem to develop earlier than the sensory ones.

A feature which is not readily understoood is the temporary enzymic hyperactivity during development in some nuclei, especially in the nucleus coeruleus. The possible relationship of this phenomenon to the deposition of melanin has been mentioned.

Of interest is the later increase of SDA in the neuropil than in the cell bodies. FLEXNER *et al.* (1941) have shown that the spacing of the neurones in the pig cerebral

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cortex is strikingly accentuated between the 90th and 180th days of gestation, coinciding with an increase in oxygen consumption. This spacing of the neurons probably reflects the development of cell processes and neuropil. That the metabolic activity of the neuropil generally occurs later than that of the cell bodies is shown by the behaviour of succinic dehydrogenase in the developing brain. Expecially the so-called molecular layers are very delayed in development. This may imply a higher functional importance of the neuropil than of the cell bodies. It does not seem reasonable to attribute the described increase of SDA to growth rather than to development, for a step by step establishment of the permanent adult distribution was found in almost any nucleus.

The changing pattern in the distribution of succinic dehydrogenase during development may be of particular value in understanding pathological processes of the developing brain. A general difference of the developing brain's resistance and mode of reaction to various stresses is known and this has been demonstrated many times. However, the differing specific metabolic pattern of the nuclei of the developing brain compared to the adult brain should be stressed. For instance, damage to the basal ganglia in early childhood and in adults does refer to metabolically quite different structures. The unique patchy distribution of SDA in the developing putamen of rat is found only during the first 10 days. This patchy pattern resembles the status marmoratus of human neuropathology which results from damage to this area in early development. Different distribution of SDA in the cerebellar and cerebral cortex might reflect in the various features of damage resulting from injuries at different periods of development.

Since the general distribution of succinic dehydrogenase in various species seems fairly constant (SHIMIZU and MORIKAWA, 1957), its pattern of development may reasonably be assumed to be similar among species. Thus, our understanding of the mode of reaction of the developing brain and the distribution of damage resulting from an injury may gain considerably from knowledge of the metabolic differences or similarities among the brain nuclei at different stages of development as revealed by histochemical techniques.

SUMMARY

The increase of succinic dehydrogenase activity in the gray matter of rat brain is demonstrated histochemically during the first twenty days *post partum*. The onset of increasing enzyme activity for most of the brain's nuclei is described, and this onset seems a rather specific characteristic of any nucleus, although it generally occurs earlier in the caudal than in the rostral portions of the brain. The enzyme activity appears earlier in the cell bodies than in the neuropil, and some regions show a complicated development, passing through several stages of enzyme distribution. Such a complicated histochemical development is found especially in the cerebral and cerebellar cortices. Some other nuclei show a unique behaviour during development, such as a temporary enzymic hyperactivity in the nucleus coeruleus. The increase of succinic dehydrogenase activity is considered a parameter of the onset of an oxidative metabolism.

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HISTOCHEMICAL INVESTIGATIONS ON SUCCINIC DEHYDROGENASE IN THE CENTRAL NERVOUS SYSTEM—II

ATLAS OF THE MEDULLA OBLONGATA OF THE GUINEA PIG

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INTRODUCTION

THE distribution of succinic dehydrogenase activity (SDA) in brain tissue of rodents has been reported recently in a fundamental study by SHIMIZU and MORIKAWA (1957). In the present report a detailed description of the nuclear pattern in the medulla oblongata of the guinea pig is presented as defined by the demonstration of succinic dehydrogenase within nervous tissue. The results were obtained by utilizing an improved cutting technique (FRIEDE, 1958) which minimizes traumatic effects to the tissue and allows the demonstration of SDA in fine and delicate structures, in particular, the neuropil. In some instances, the capability of the technique to demonstrate the minute differences between subnuclei definitely exceeds the results obtained by the usual cytoarchitectonic techniques. Further observations will show that even differences between cortical areas are quite clear with the SDA technique.

It is hoped that the microphotographs of sections of the medulla oblongata presented in this paper together with a detailed description of the nuclei will provide a mapping of normal activity, useful as a base in neuroanatomy, neurophysiology and experimental neuropathology. Importance should be attributed to the fact that the histochemical technique employed offers a method for demonstrating the neuropil, a part of the nervous system which offers very poor access to the conventional techniques of neurohistology.

The nomenclature and arrangement of this atlas corresponds to that of the cytoarchitectonic atlas of the rhombencephalon of the rabbit by MEESSEN and OLSZEWSKI (1949). Thus, the plates of this report may readily be compared with the plates of the cytoarchitectonic atlas of these authors. The cytoarchitecture of the medulla oblongata of the guinea pig is not dealt with in the present article, since it is very similar to that of the rabbit.

MATERIAL AND METHODS

The histochemical technique employed in this investigation is essentially the same as that used in the first study of this series (FRIEDE, 1959). The animals were killed by a blow on the neck, and the brains were removed as quickly as possible. Unfixed frozen sections, 60 μ thick, were employed for demonstrating SDA by the tetrazolium technique described in the first article. For a detailed description of this

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technique see also FRIEDE (1958). The sections are mounted in glycerine–gelatine, and no counterstaining was used.

Complete serial sections were made of the entire medulla oblongata of three young adult guinea pigs. This paper is based principally on an examination of these three series. In addition, a large number of random sections from other incomplete series were available for comparison.

RESULTS

The SDA reaction results in a deposition of minute diformazan granules of equal size and dark violet colour. Besides this granular reaction, the background is found stained diffusely yellow or orange, especially in the white matter which usually shows no granular reaction at all. This background staining without granular staining will be termed a negative reaction, although it should be understood that a chemically different reaction is involved (BURTNER, BAHN and LONGLEY, 1957). The positive reaction of the grey matter appears as a light, dark, or intense violet stain corresponding with the amount of deposited formazan granules. Thus weak, mild, or strong SDA may be described from the intensity of the stain. The new 'Nitro BT' (NACHLAS *et al.*, 1957) showed identical distribution of SDA in comparative studies.

The reaction of the cell bodies is independent from that of the neuropil. Nuclei are found with strong activity in the cell bodies and none in the neuropil, as well as nuclei with strong activity in both the cell bodies and the neuropil. Intermediate stages between these extremes may also be seen. Moreover, the morphological appearance or distribution of the SDA reaction in the neuropil varies: (1) uniformly distributed granules give a *diffuse* arrangement; (2) an irregular distribution shows as a *cloudy* or *scattered* arrangement; (3) and organization of the granules produces a *reticular* arrangement. The activity seen in the dendrites in this latter arrangement frequently blends into that of the adjacent neuropil, so that sharp distinction of either is impossible.

In the following description of the results, the plates are described in caudo-cranial sequence. A full description of a certain nucleus is given only with the plate optimally demonstrating the nucleus; a brief reference is provided with other plates exhibiting the same nucleus.

Plate I

The section is taken through the pyramidal decussation at the level of the most caudal portion of the hypoglossal nucleus.

Nucleus tractus spinalis trigemini caudalis (*N.V.sp.c.*) Subnuclei magnocellularis (*mc*), gelatinosus (*g*) and zonalis (*z*): The histochemical differences between all the nuclei of the trigeminal pathways are significant and interesting. The magnocellular part of this nucleus shows strong SDA in the neuropil in which cell bodies of somewhat stronger SDA are visible. The texture of the neuropil is irregular. In contrast the subnucleus gelatinosus shows a uniform distribution of exceedingly strong SDA without visible cell bodies. This SDA decreases towards the periphery where the nucleus is bordered by the subnucleus zonalis. Cell bodies and neuropil of this latter subnucleus also exhibit strong SDA.

Nucleus reticularis medullae oblongatae. subnucleus ventralis (*R.v.*) and dorsalis (*R.d.*): The subnucleus ventralis exhibits strong SDA in the cell bodies but weak SDA

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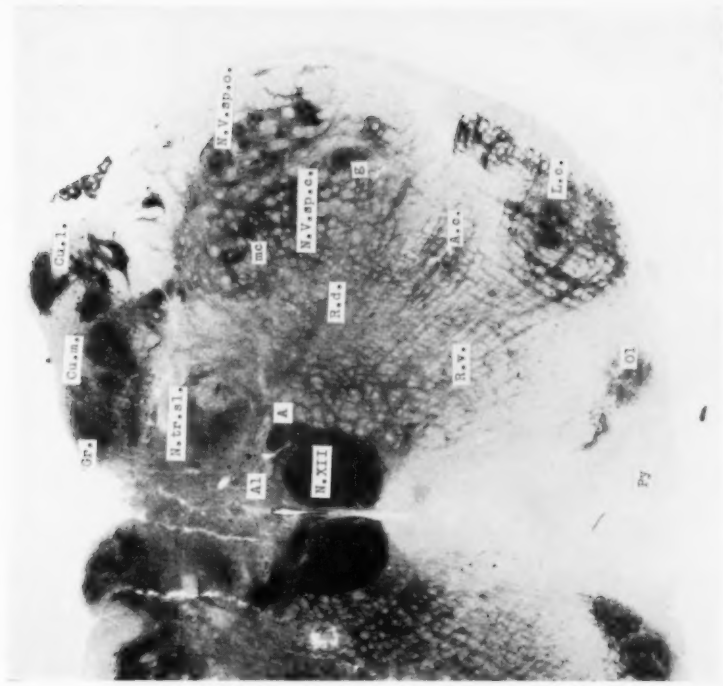

Plate II.

Plate I.

Plate III.

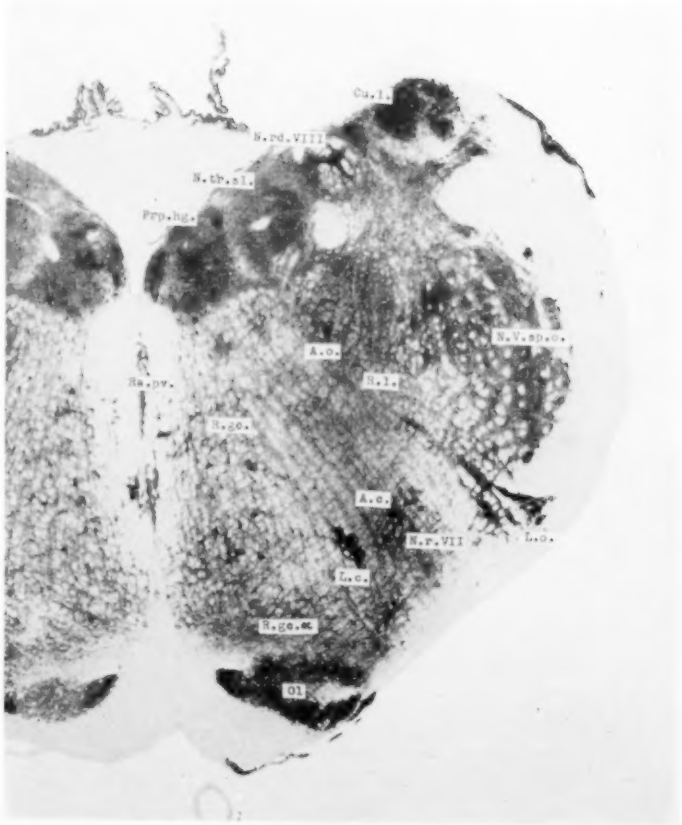

Plate IV.

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Plate V.

Plate VI.

Plate VII.

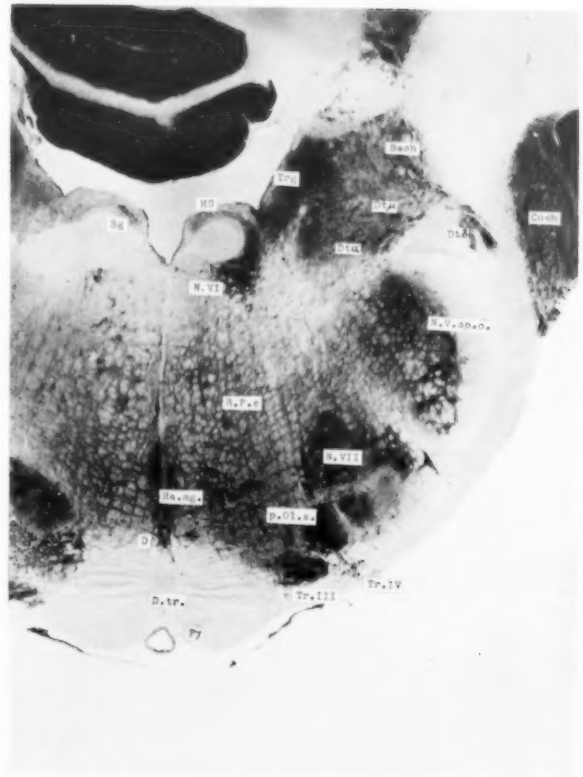

Plate VIII.

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Plate IX.

Plate X.

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in the neuropil. The dendrites of the cell bodies are seen to extend over vast distances. In the subnucleus dorsalis, however, the cell bodies stain less intensely, but their neuropil stains more strongly, rendering the outlines of the cell bodies indistinct. The SDA of the neuropil has a reticulated texture.

Nucleus residualis cornu anterioris (*r.Co.a.*). Among this group of persisting cells of the ventral horn type the neuropil has a stronger SDA than that in the adjacent nucleus reticularis. The SDA of the cell bodies is also very strong.

The *nucleus hypoglossus* (*N.*XII) seen here in its most caudal portion exhibits strong SDA in the cell bodies and weak SDA in the neuropil. This distribution differs from the appearance of this nucleus at higher levels of the medulla (Plate II). At the dorsolateral surface of the nucleus hypoglossus an area of strong SDA in the neuropil is found which is obviously identical with the *cell group A* (*A*) of MEESSEN and OLSZEWSKI (1949). The nuclei *alaris* (*Al*), *tractus solitarii* (*T.sl.*), *gracilis* (*Gr.*), and *cuneatus medialis* (*Cu.m.*) are also visible but will be described with Plate II.

Plate II

The section is taken at the midpart of the hypoglossal nucleus and includes the most caudal portion of the inferior olivary nucleus.

Nucleus hypoglossus (*N.*XII). This nucleus has a strong SDA in the neuropil, decreasing sharply at the borders of the nucleus. The SDA in the cell bodies is slightly stronger than that in the neuropil. The most caudal part of the nucleus exhibits decreasing SDA of the neuropil but strong SDA of the cell bodies (Plate I).

Nucleus of Roller (*Rol.*). Stronger SDA of the neuropil clearly separates the nucleus of Roller from the nucleus hypoglossus.

Nucleus tractus solitarii (*N.tr.sl.*). The nucleus tractus solitarii belongs to a group of nuclei with unusually weak SDA. The subnucleus parvocellularis shows a weak, diffuse arrangement of SDA. This weak SDA is most strikingly in Plate V by its contrast with the stronger SDA at the borders of the nucleus triangularis and nucleus prepositus hypoglossi. In the magnocellular part of the nucleus tractus solitarii the SDA is mild, and single cell groups in the vicinity of the tractus solitarius show clearly by their stronger SDA (Plate III).

Nucleus alaris or dorsalis vagi (*Al*). Although this nucleus is outlined as a separate cell group only at more rostral levels (Plate III), its scattered cells are easily identified in other sections because of their exceedingly weak SDA. The nucleus alaris and the nucleus tractus solitarii show the same weak SDA in both the cell bodies and the neuropil.

Nucleus gracilis (*Gr.*) *and cuneatus medialis* (*Cu.m.*). These nuclei differ markedly from the motor nuclei of the cranial nerves by their lower SDA. The neuropil shows weak SDA in the nucleus gracilis and slightly more SDA in the nucleus cuneatus medialis. This difference is less pronounced rostrally than caudally (Plate III). The cell bodies in these nuclei exhibit stronger SDA than the neuropil.

Nucleus cuneatus lateralis (*Cu.l.*). SDA in this nucleus is stronger than in the nucleus cuneatus medialis and is similar to that in the motor nuclei. Single cell bodies with exceedingly strong SDA are found at the borders of the nucleus, just outside of the intensely staining neuropil.

The distribution of SDA in the *nucleus reticularis* (*R.v.* and *R.d.*) is the same as described in Plate I. The cell group *A* (*A*) is also still visible. More laterally the

medial portions of the *nucleus tractus spinalis trigemini caudalis* and the lateral portions of the *nucleus tractus spinalis trigemini oralis* seem to form one single complex. With high-power magnification the differentiation of these two parts of the nucleus tractus spinalis trigemini is possible by the differences in the texture of the neuropil. The oral portion of this nucleus will be described with Plate IV.

Nucleus ambiguus caudalis (*A.c.*). This nucleus exhibits a stronger SDA in the neuropil than the surrounding nucleus reticularis and has a distinct reticular appearance. It lacks a sharply defined border. The cell bodies and dendrites are outlined by strong activity.

Nucleus lateralis caudalis (*L.c.*). Distribution and intensity of SDA in this nucleus is similar to that in the motor nuclei of the cranial nerves. The subnucleus parvocellularis is identified by its clearly visible smaller cells.

The *nucleus olivaris inferior* (*Ol*) of which the caudal pole is visible will be described in Plate III.

Plate III

The level of the section is determined by the enlargement of the central canal where it joins the fourth ventricle.

The *ependyma* of the canalis centralis is deeply stained in contrast to the subependymal glial tissue. The glial cushion at the obex, *promontorium gliosum* (*Pr.gl.*), sometimes termed the area postrema, exhibits extremely weak SDA. This weak reaction was found in all the sections examined, but in our experience the low enzyme content of this area seems not to be constant among individuals of other species.

The *nuclei hypoglossus* (*N.*XII), *of Roller* (*Rol.*) and the *cell group A* (*A*) show the same shape and SDA content as in Plate II. Dorsally to the nucleus hypoglossus is seen the *nucleus intercalaris* (Staderini) (*Ic*). The distribution of mild or weak SDA in this nucleus is similar to that of the adjacent *nuclei alaris* (*Al*) and *tractus solitarii* (*T.sl.*). The SDA in the neurones of the nucleus alaris is always concentrated in the perinuclear zone and adjacent to the cell membrane. The *nucleus alaris* or *dorsalis vagi* (*Al*) forms an easily discernable cell group at this level. *Nuclei gracilis* (*Gr*), *cuneatus medialis* (*Cu.m.*), and *cuneatus lateralis* (*Cu.l.*) appear the same as in Plate II. The *nucleus lateralis caudalis* (*L.c.*) also has little changed. More laterally the *nucleus lateralis oralis* (*L.o.*) comes in view, exhibiting strong SDA of its cell bodies with a scattered arrangement of the neuropil. The transition of the *nucleus ambiguus caudalis* (*A.c.*) into the *nucleus retrofacialis* (*r.*VII) and the features of the *nucleus tractus spinalis trigemini oralis* (*N.V.sp.o.*) will be described in Plate IV.

The *nucleus reticularis medullae oblongatae* still exhibits the typical features of its ventral (*R.v.*) and dorsal (*R.d.*) portions, as described for Plate I. Adjacent to the ventrolateral surface of the nucleus hypoglossus, the SDA of the neuropil is increased, separating this portion from the remainder of the nucleus reticularis medullae oblongatae. Another interesting feature is seen in the ventromedial portion of the nucleus reticularis medullae oblongatae: Irregularly shaped cell groups, defined by strong SDA in the neuropil, are found in the vicinity of the raphe. These groups show a considerable individual variation, even between the right and left halves of the medulla oblongata. Sometimes they appear connected with the *nucleus raphes parvus* through equal SDA in the intervening neuropil. This connexion is seen to be nearly

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complete in Plate IV. Since these cell groups are separated from the rest of the subnucleus ventralis only by the strong SDA of their neuropil, they are not discernable with common staining techniques. Only in these afore mentioned cell groups and in the nucleus raphes magnus was an individual variation in the distribution of the neuropil found, resulting in various nuclear configurations.

Nucleus parolivaris medialis, dorsalis and olivaris principalis (*Ol*). These nuclei display strong SDA in both the neuropil and their small cell bodies, giving the appearance of a broad band of diffuse reaction. No characteristic differences between the subnuclei are evident.

Plate IV

The section is taken from the level of the oral portion of the inferior olivary nucleus.

Nucleus prepositus hypoglossi (*Prp.h.*). The SDA in this nucleus is definitely weaker than that in the nucleus hypoglossus. One quite interesting feature is the tendency of irregular cell groups to form through concentration of SDA within the nucleus. The feature of these cell groups seems not constant.

The *nucleus alaris* or *dorsalis vagi* (*Al*) and nucleus *tractus solitarii* (*T.sl.*) are well outlined by their typically weak SDA. More laterally, the nucleus gracilis is replaced by the *nucleus radicis descendentis nervi vestibularis* (*N.rd.*VIII) (spinal vestibular nucleus), described in Plate V. The *nucleus cuneatus lateralis* (*Cu.l.*) is reduced in size but otherwise unchanged.

Nucleus ambiguus oralis (*A.o.*). The distribution of SDA in this nucleus is very similar to that of the nucleus ambiguus caudalis (Plate II). Since the borders of the activity in the neuropil of the nucleus ambiguus oralis are indistinct, the nucleus appears to be a part of the nucleus reticularis dorsalis whose neuropil is only somewhat less intensely stained.

Nucleus tractus spinalis trigemini oralis (*N.V.sp.o.*). The distribution of SDA in this nucleus suggests a division into ventral and dorsal parts. In the ventral part SDA in the neuropil is weak, but the large star-shaped cell bodies exhibit strong activity. The dorsal part has stronger SDA of the neuropil with less pronounced outlining of cell bodies. Sometimes the contrast between these two divisions of the nucleus is so marked that they appear as two distinct nuclei.

Nucleus reticularis gigantocellularis (*R.gc.*) *and pars* α (*R.gc.* α). This part of the formatio reticularis shows almost no neuropil, but the giant cells and their long dendrites are demonstrated by an exceedingly strong SDA. The ventrally situated pars α of the nucleus gigantocellularis exhibits a reticular arrangement of mildly staining neuropil.

Nucleus reticularis lateralis (*R.l.*). This nucleus is a continuation of the more caudal nucleus reticularis dorsalis. The distribution of SDA is nearly the same in both.

Nucleus raphes parvus (*Ra.pv.*). This nucleus shows strong SDA in the cell bodies and in the neuropil; frequently it is found asymmetrical to the midline (Plates VII to IX). Connexions with the previously mentioned individual cell groups comprising the ventromedial portion of the nucleus reticularis medullae oblongatae are observed.

Nucleus retrofacialis (*N.r.*VII). Demonstrated by means of usual histological techniques, the nucleus retrofacialis has a very intimate relation with the nucleus ambiguus caudalis. With the tetrazolium technique, the borders between these nuclei

appear sharp, especially to high power magnification. The neuropil of the nucleus ambiguus caudalis appear reticular, but the nucleus retrofacialis exhibits a diffuse distribution of SDA in the neuropil. The cell bodies of the nucleus retrofacialis react more strongly and are outlined sharply giving a texture of greater contrast than is found in the nucleus ambiguus caudalis.

Portions of the *nuclei lateralis oralis* (*L.o.*) and *caudalis* (*L.c.*) and the nucleus *olivaris inferior* (*Ol*) show the same distribution of SDA as described in Plate IV.

Plate V

The level of this plate is indicated by the appearance of the most caudal portion of the acoustic tubercle and the facial nucleus.

Nucleus radicis descendentis nervi vestibularis (*N.rd.*VIII). Strong SDA is present in the neuropil as well as in the cell bodies. Although intense staining of the neuropil usually gives homogenous appearance to the nuclei of the medulla oblongata, this nucleus is seen frequently perforated by descending fibre bundles.

Nucleus triangularis (*medial vestibular nucleus*) (*Trg.*). SDA is strong and uniformly distributed throughout the neuropil of the nucleus triangularis, the cell bodies being barely visible.

The *nucleus prepositus hypoglossi* (*Prp.hgl.*) is discernable from the nucleus triangularis by its somewhat weaker SDA. The weak SDA of the *nuclei tractus solitarii* (*T.sl.*) and *alaris* (*Al*) contrasts to the strong SDA of the surrounding nuclei. Only the caudal portion of the *nucleus facialis* (VII) is identifiable here, and a full description of the nucleus accompanies Plate VI. The ventral portion of the medulla oblongata was considered with Plate IV. *Cell group E* (*E*) of MEESSEN and OLSZEWSKI (1949) appears as a small area of strong SDA.

Plate VI

The level of this plate is indicated by a midsection through the facial nucleus.

Nucleus facialis (VII). The facial nucleus shows strong and diffuse SDA in the neuropil and even stronger SDA in the cell bodies, a distribution similar to that of the nucleus hypoglossus. The border of the nucleus is sharp at its ventral surface, but shows reticular connexions in the dorsal area.

Cell group F (*F*) *of* MEESSEN *and* OLSZEWSKI (1949). This cell group shows extremely strong SDA in both the cell bodies and the neuropil. Besides the first layer of the tuberculum acusticum, this group shows the strongest reaction in this section. This seems surprising, because of the unknown function of the cell group.

The remainder of the nuclei were already discussed in Plate V. Below the nucleus facialis the *subnucleus trapezoides* IV (*Tr.*IV) appears; the complex of the nucleus trapezoides will be discussed in Plate VII.

Plate VII

The section is made through the middle of the acoustic tubercle.

Tuberculum acusticum (*Tub.ac.*). A more detailed subdivision of the layers of the tuberculum acusticum is shown by SDA than by conventional staining techniques. The first (I, molecular) layer exhibits extremely strong, diffusely arranged SDA. The second (II) layer has a slightly weaker and irregularly arranged SDA. The third (III) layer is split into an upper lamina of mild SDA in the neuropil and cell

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bodies and into a deeper lamina of strong SDA in the cell bodies but weak SDA in the neuropil. This subdivision of the third layer is not evident in common neurohistological techniques.

Nucleus nervi cochlearis (*Coch*). This nucleus shows strong SDA in the cell bodies with short indistinct dendrites. The SDA of the neuropil is stained consistently more intensely in the dorsal part than in the ventral, thus subdividing the nucleus.

Subnuclei of Deiters α *to* δ (*Dt* α *to* δ) (*Lateral vestibular nucleus*). The nucleus of Deiters demonstrates the capability of the tetrazolium technique to depict architectonic details. The appearance of the subnuclei α and γ is similar, showing the typically large, star-shaped Deiter's cells. The cells are strongly stained and the dendrites are visible over vast distances. The neuropil, however, exhibits weak activity. The subnucleus β could easily be mistaken as a part of the nucleus triangularis. The SDA in the neuropil of this subnucleus almost matches the amount of activity in the nucleus triangularis. No distinct boundary between the two can be made out, and the big Deiter's cells in the subnucleus β must be searched for to positively identify the subnucleus. The subnucleus δ has mild SDA of the neuropil, exhibiting a reticular texture, and appears to join the tuberculum acusticum.

The *nucleus tractus spinalis trigemini oralis* (*N.V.sp.o.*) and the nucleus *reticularis gigantocellularis* (*R.gc.*) are described with Plate IV, and the *nucleus facialis* (VII) with Plate VI.

Nucleus parolivaris superior (*p.Ol.s.*). The lancet shaped cells of this nucleus show strong SDA extending far out into the cell processes at both ends of the cell. Since the SDA of the neuropil is weak, the approximately parallel situated cell processes give an unique striped pattern.

Subnuclei trapezoides I *to* V (*Tr* I *to Tr* V). These subnuclei are another example of the demonstration of delicate topographical differences between subnuclei by the tetrazolium technique, providing distinctly superior results to those obtained by customary cytoarchitectonic methods. The SDA distribution outlines clearly five subnuclei, herein numbered subnuclei I to V. These subnuclei border the nucleus olivaris and parolivaris superior at the ventromedial surface. The cell bodies have equally strong SDA in all subnuclei, but the neuropil shows mild SDA in the subnuclei II and IV and lacks SDA in the subnuclei I, III, and V. The subnucleus IV is situated most caudal and best seen in Plate VI.

Plate VIII

Although not of the same series as the remainder of the sections (it is cut inclined in comparison to the others), this plate was included because of its excellent demonstration of the features of the large nucleus of the raphe and the caudal extension of the pontine reticular formation. The level of the plate is identified by the abducens nucleus.

Nucleus abducens (*N.*VI). The intensity and distribution of SDA match that of the facial nucleus. The borders of the neuropil are sharp dorsally towards the facial nerve but irregular ventrally.

Nucleus reticularis pontis caudalis (*R.pc.*). Although this nucleus is called pontine, its most caudal portion extends into the medulla oblongata. The SDA arrangement continues the pattern of the most caudal part of the reticular formation, specifically the nucleus reticularis medullae oblongatae.

Nucleus raphes magnus (*Ra.mg.*). This nucleus does not appear as an entity with SDA technique. Strong SDA in the neuropil connects the nucleus on both sides with the adjacent halves of the nucleus reticularis pontis caudalis, thus forming a large complex. The mode of connexion of these nuclei varies considerably through differences in the distribution of SDA in the neuropil.

Cell group D of MEESSEN *and* OLSZEWSKI (1949) (*D*). The cell group *D* can be recognized at the ventral circumference of the nucleus raphes magnus. Strong SDA is found in the small cell bodies of this nucleus, but the neuropil exhibits only weak activity, definitely separating the cell group *D* from the nucleus raphes magnus.

Trapezoid decussation (*D.tr.*). The fibres of the trapezoid decussation show an interesting and exceptional behaviour. Whereas white matter generally shows a negative reaction, these fibres exhibit a distinct granular one. In no other fibre system of the adult guinea pig could a similar reaction be demonstrated.

Plate IX

The level of the plate is through the middle of the superior olivary nucleus.

Nucleus supragenualis (*Sg*). This is a small cell group with mild SDA in the neuropil as well as in the cell bodies. The SDA in this nucleus is distinctly stronger than that in the surrounding central grey matter.

The *central grey matter* exhibits weak SDA of the neuropil and no cell bodies are visible. The SDA content of the neurones of the central grey matter, therefore, must be very low.

Nucleus of Bechterew (*superior vestibular nucleus*) (*Bech*). This nucleus is characterized by typically cloudy or scattered mild SDA in the neuropil. The cell bodies are readily seen through their stronger reaction.

Medially from the nucleus of Bechterew appears the most rostral portion of the *nucleus triangularis* (*Trg.*). The nucleus tractus spinalis trigemini oralis is replaced by the *nuclei trigemini sensibilis principalis* (*N.V.sens.princ.*) and *trigemini motorius* (*N.V.mt.*) which will be described with Plate X. The *nucleus reticularis pontis* was already described with Plate VIII.

Nucleus olivaris superior (*Ol.s.*). The appearence of this nucleus is very similar to the nucleus olivaris inferior, exhibiting strong SDA both in the neuropil and in the cell bodies. Medially from the nucleus olivaris superior the *subnuclei trapezoides* (*Tr.* I to *Tr.* V) are seen which are described with Plate VIII.

Plate X

The section is made at the level of the trigeminal motor nucleus. The *nucleus supragenualis* (*Sg*) and the *nucleus of Bechterew* (*Bech*) are still visible. Laterally, the *nucleus parabrachialis* appears (*P.Br.*).

Nucleus trigemini mesencephalicus (*N.V.mes.*). This nucleus is outstanding in showing extremely strong SDA in the cell bodies and in completely lacking it in the neuropil.

Nucleus trigemini motorius principalis (*N.V.mt.*). Like other motor nuclei, this nucleus has strong activity in the neuropil and even stronger activity in the cell bodies. The reaction in the neuropil is diffuse, but transfixing fibre bundles result in a gross perforated appearence. Subdivisions of the nucleus are not discernible.

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Nucleus trigemini sensibilis principalis (*N.V.sens.princ.*). Under high power magnification, the histochemical structure of this nucleus is different from that of the nucleus spinalis trigemini oralis. The pattern of the former shows more contrast with small, round, strongly staining cell bodies in a somewhat scattered arrangement of less intensely stained neuropil. The neuropil of the nucleus spinalis trigemini oralis appears more diffuse or reticular and the reaction in the cell bodies is less pronounced.

Cell group I (*I*) of MEESSEN *and* OLSZEWSKI (1949). Large cells and a SDA distribution similar to that of the nucleus trigemini motorius mark this group which is located within the nucleus trigemini sensibilis.

Surrounding the trigeminal nuclei is the *regio H* (*H*) of MEESSEN and OLSZEWSKI (1949), characterized by mild SDA of the neuropil.

DISCUSSION

SDA is involved in the Krebs cycle and forms an important link in the oxidative breakdown of carbohydrates, proteins and lipids. The enzyme appears very intimately related to cytochrome oxidase, for both enzymes are accumulated in the mitochondria (HOGEBOOM, CLAUDE and HOTCHKISS, 1946). In fractional centrifugates of homogenates of the rat brain, SDA appears in 78 per cent in the mitochondria, in 16 per cent in the cell nuclei, and in 5 per cent in the supernatant fraction (ABOOD *et al.*, 1952). The histochemical distribution of SDA and cytochrome oxidase in brain tissue is almost identical (SHIMIZU, MORIKAWA and ISHI, 1957). In the present investigation, random sections prepared both for cytochrome oxidase by the labile Nadi reaction and for succinic dehydrogenase also did not show any differences in the distribution of the enzymes. Because of the biological importance of SDA and its intimate linkage to cytochrome oxidase, SDA can be considered a parameter of oxygen consumption and the general functional activity of tissue (HIMWICH, 1951; SCHIEBLER, 1955). The transformation of succinic acid to fumaric acid by the succinic dehydrogenase is one of the most rapid biochemical reactions and was assumed by HOAGLAND (1948) to be a pacemaker for the electroencephalographic displays. Regarding the above mentioned facts, the plates of this atlas might constitute a measure of the general metabolic activity of the brain stems nuclei.

The histochemical distribution of SDA in brain tissue has been studied already by several authors (LEDUC and WISLOCKI, 1952; PADYKULA, 1952; RUTENBERG, WOLMAN and SELIGMAN, 1953; NEUMANN and KOCH, 1953; MUSTAKALLIO, 1954; SHIMIZU *et al.*, 1957; SHIMIZU and MORIKAWA, 1957; FRIEDE, 1957). Apart from some disparity as to finer details, SDA has been found by all the investigators to be distributed in the same general pattern. The pattern of SDA distribution in rodents was fairly constant in a comparative study (SHIMIZU and MORIKAWA, 1957). Samples of rat, dog, pig and cat brain, used in the present investigation for comparison, displayed similar results to those obtained in the guinea pig. However, minor differences of SDA distribution in some nuclei may be valuable in comparative anatomy; for example, the subdivision of the nuclei trapezoides described herein is less pronounced in the rat but is about the same in the cat.

The quantitative accuracy of the technique used in this investigation proved satisfactory by comparison with the results of the biochemical determination of the SDA content of different nuclei of the dog's brain (BURGEN and CHIPMAN, 1951).

The intensity of staining showed the same variation among the different nuclei that the biochemical analysis revealed.

The advantage of the technique employed in the present investigation is the finer demonstration of SDA in the neuropil. Which morphological elements are responsible for this activity is not clear. Since a gradual transition is observed between the dendrites and the neuropil, dendrites and possibly even synapses may contain most of the SDA. In some nuclei, such as the nucleus reticularis, a reticular texture is given to the neuropil by the network of easily recognized, thick dendrites. In other nuclei, short dendrites are seen to blend into a cloudy neuropil, and in still other nuclei cell bodies are found in a diffuse neuropil without recognizable dendrites. Therefore, it is concluded that the appearance of the neuropil might be a function of dendrite branching. No indication was found for SDA in glial cell bodies.

The apparently independent SDA content of the cell bodies and surrounding neuropil is of interest. It seems reasonable to predict an integrative function through synapses where the neuropil shows strong SDA, but merely efferent functions in areas which show SDA exclusively in cell bodies. This hypothesis is suggested by the frequent subdivision of a nucleus into a portion with SDA prevailing in the neuropil and a portion with SDA exclusively in the cell bodies. Especially in such structures as the cerebral cortex (not described in this article) and the acoustic tubercle such subdivision is evident in upper, integrative layers with strong SDA in the neuropil and into deeper, efferent layers with SDA only in cell bodies.

Current investigations on the SDA content of isolated cells have shown a definitely lower SDA in cell bodies than in the surrounding neuropil in some areas of the brain. The granular layer of the cerebellum, for example, almost completely lacks SDA in its granular cells and all enzyme activity is concentrated in the synaptic glomeruli between them. Thus, in some areas the metabolic activity of the neuropil seems definitely to exceed that of the cell bodies. This agrees with the lack of a direct proportionality found by DUNNING and WOLFF (1936, 1937) between vascularization and the number and size of cell bodies. The mitochondria in the neuropil rather than the density of the cell bodies seem to be a parameter of the vascularity (SCHARRER, 1945). Planimetric studies on the cerebellar cortex have shown the dendrites and the neuropil of the molecular layer to be parameters for the cell density and thickness of the other cortical layers (FRIEDE, 1955).

Any discussion of the physiological implications of the differential distribution of succinic dehydrogenase in the brain stem's nuclei is beyond the limit of this article, but three exceptional findings are of special interest.

The very weak SDA in the nucleus tractus solitarii, nucleus dorsalis vagi and nucleus intercalatus, which also has been described by SHIMIZU and MORIKAWA (1957), raises the question of the physiological importance of this finding. Only further investigations by different histochemical techniques will provide information as to whether the slight activity found in these nuclei reflects a generally low metabolic rate or an unknown specific metabolic process. The nuclei supraopticus and paraventricularis, for example, have a very low SDA in their neurones and these nuclei are known to have a neurosecretory function. The detection of an emetic trigger zone supplied with chemoreceptors in the region of the above mentioned nuclei with low SDA content might be one valuable lead (BORRISON and WANG, 1951).

The formation of individual cell complexes in the nucleus reticularis medullae

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oblongatae and the nucleus raphes magnus might be of physiological importance. These cell groupings are defined only by SDA in the neuropil and thus are indiscernible by conventional staining techniques. There was no indication found for an explanation of these findings other than a true variability of the regions.

The presence of SDA in the fibres of the trapezoid decussation differs from that in white matter. This abnormal behaviour is possibly a phylogenetic trait, since in any nerve fibre of the leech strong SDA may be found. Another possible and intriguing explanation could be that the activity reflects the extreme paucity of oligodendroglia in this fibre system as found in our current cell countings.

It is intended to continue these investigations to complete a mapping of the entire brain of the guinea pig. Studies now in progress indicate a rich subdivision of the thalamus and differences between cerebral cortical areas by distribution of SDA.

SUMMARY

The histochemical distribution of succinic dehydrogenase activity in the medulla oblongata of the guinea pig was investigated by an improved tetrazolium technique which more adequately demonstrates the distribution of the enzyme even in the most delicate structures, such as dendrites and the neuropil. A mapping of the enzyme distribution, following the form of an atlas, is presented, and finer differences of all the nuclei of the medulla oblongata are described. Some areas were found to exhibit very exceptional behaviour: the nucleus tractus solitarii and dorsalis vagi contain very little of the enzyme; the ventromedial portion of the nucleus reticularis and the nucleus raphes magnus tend to form individually shaped cell complexes or subdivisions which are defined only by an accumulation of succinic dehydrogenase in the neuropil; and the fibres of the trapezoid decussation give a positive reaction unlike other fibre systems.

The amount and distribution of succinic dehydrogenase in the cell bodies and the neuropil is typical and constant for any nucleus and forms specific patterns in some of the nuclei. However, no general relationship was found between the amount of enzyme contained by the cell bodies and the surrounding neuropil. The possible physiological implications of this independent behaviour are discussed. Considering the known biochemical importance of succinic dehydrogenase in metabolism, the presented atlas may be regarded as mapping the general activity of the nuclei of the medulla oblongata.

INDEX OF ABBREVIATIONS AND SYNONYMS

r.Co.a.:	Nucleus residualis cornu anterioris (anterior grey column).
*N.*XII:	Nucleus hypoglossus.
Rol.:	Nucleus of Roller.
A.c.:	Nucleus ambiguus caudalis.
A.o.:	Nucleus ambiguus oralis.
*N.r.*VII:	Nucleus retrofacialis.
*N.*VII:	Nucleus facialis.
*N.*VI:	Nucleus abducens.
N.V.mt.:	Nucleus trigemini motorius.
Gr.:	Nucleus gracilis.
Cu.m.:	Nucleus cuneatus medialis.

Cu.l.: Nucleus cuneatus lateralis.
N.V.sp.c.: Nucleus tractus spinalis trigemini caudalis.
mc: Subnucleus magnocellularis.
g: Subnucleus gelatinosus.
z: Subnucleus zonalis.
N.V.sp.o.: Nucleus tractus spinalis trigemini oralis.
N.V.sens.princ.: Nucleus trigemini sensibilis principalis.
N.V.mes.: Nucleus trigemini mesencephalicus.
Coch: Nucleus nervi cochlearis.
Tub.ac.: Tuberculum acusticum.
*N.rd.*VIII: Nucleus radicis descendentis nervi vestibularis (Spinal vestibular nucleus).
Trg.: Nucleus triangularis (Medial vestibular nucleus).
Dt.: Nucleus Deiters (Lateral vestibular nucleus).
Bech: Nucleus Bechterew (superior vestibular nucleus).
Ol: Nucleus olivaris principalis and parolivaris (inferior olivary nucleus).
Ol.s.: Nucleus olivaris superior.
p.Ol.s.: Nucleus parolivaris superior.
Tr I *to Tr* V: Subnuclei trapezoidei I to V.
D.tr.: Fibres of the trapezoid decussation.
N.tr.sl.: Nucleus tractus solitarii.
Al.: Nucleus alaris (Dorsal motor nucleus of vagus).
Ic.: Nucleus intercalatus (Staderini).
L.o.: Nucleus lateralis oralis (Nucleus of lateral funiculus of WINKLER and POTTER).
L.c.: Nucleus lateralis caudalis.
Prp.hg.: Nucleus prepositus hypoglossi.
Sg.: Nucleus supragenualis.
Nucleus reticularis medullae oblongatae (reticular substance).
R.v.: Subnucleus ventralis.
R.d.: Subnucleus dorsalis.
R.gc.: Nucleus reticularis gigantocellularis (magnocellular nucleus of reticular formation).
R.gc. α: Pars α of the nucleus reticularis gigantocellularis.
R.l.: Nucleus reticularis lateralis.
R.P.c.: Nucleus reticularis pontis caudalis.
P.P.c. α: Pars α of the nucleus reticularis pontis caudalis.
Ra.pv.: Nucleus raphes parvus.
Ra.mg.: Nucleus raphes magnus.
H.G.: Periventricular central grey matter (Hoehlengrau).
A,D,E,F,I,H: Cell groups *A,D,E,F,I,H* of MEESSEN and OLSZEWSKI.

Abbreviations and terminology are the same as in MEESSEN and OLSZEWSKI (1949)

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SOME OBSERVATIONS ON THE EFFECT OF D-GLUTAMATE ON THE GLUCOSE METABOLISM AND THE ACCUMULATION OF POTASSIUM IONS IN BRAIN CORTEX SLICES

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IT was reported by WEIL-MALHERBE (1938) that the aerobic glycolysis of brain cortex slices is stimulated by glutamate or related compounds. On the other hand, TERNER, EGGLESTON and KREBS (1950) showed that slices of brain cortex can accumulate potassium ions together with L-glutamate against the concentration gradient. Although the mechanism of glutamate action in these phenomena has been discussed by several authors (STRECKER, 1957; TERNER *et al.*, 1950), the present investigation was intended to correlate the metabolism of glucose in brain slices with potassium and glutamate accumulation.

In previous papers from this laboratory, the effect of L-glutamate on phosphorus metabolism (TSUKADA, TAKAGAKI and HIRANO, 1958), and the effect of changing the ionic milieu on glucose metabolism in brain slices (TAKAGAKI and TSUKADA, 1957) were reported. In continuation of these studies, the authors were interested in investigating the effect of the D-isomer of glutamate on glucose metabolism and on potassium accumulation in various ionic media, because the D-isomer was not thought to be metabolized in brain slices. It was demonstrated that in a potassium-free medium D-glutamate does not stimulate the aerobic glycolysis, and that the augmented oxygen uptake, due to the addition of 2:4-dinitrophenol or to a calcium-free medium, is completely suppressed by D-glutamate, but that induced by KCl is not inhibited. The present paper also deals with the findings that D-glutamate is less effective than its L-isomer for the accumulation of potassium ions in brain slices, but the former is taken up in the same way as the latter. The relationships between these observations are discussed.

EXPERIMENTAL

General. Slices of cerebral cortex of guinea pigs were prepared as described previously (TAKAGAKI, HIRANO and TSUKADA, 1957; TAKAGAKI and TSUKADA, 1957; TSUKADA *et al.*, 1958), and pooled in ice-cooled Ringer-solution during their preparation. The preparation of slices from both hemispheres took about 20 min. The pooled slices were then weighed on a torsion balance after blotting with a moistened filter paper to obtain the initial wet weight (water content, 84·8 ± 0·7(9)%, according to representative determinations). Incubation was carried out at 37°C under pure oxygen with constant shaking.

The medium used in the control experiments contained 0·127 M-NaCl, 0·0051 M-KCl, 0·00273 M-$CaCl_2$, 0·00127 M-KH_2PO_4, 0·00127 M-$MgSO_4$ and 0·01 M-sodium phosphate buffer of pH 7·0. Sodium-free medium was prepared by substituting choline chloride for sodium chloride; when potassium in the slices was to be determined, 0·05 M-glycylglycine was used instead of sodium phosphate buffer, and when glutamate was to be determined, potassium phosphate buffer was used. The

other media were the same as described in a previous report (TAKAGAKI and TSUKADA, 1957). Glucose was added at the concentration of 100 mg/100 ml except when otherwise stated, and L- or D-glutamate at 10 mM concentration. D-Glutamic acid used was a product of Research Laboratory of Ajinomoto Company, Ltd., Tokyo. Other chemicals were commercial 'reagent grade' and were used without further purification. Water was distilled twice and kept in a polyethylene bottle.

Chemical determinations. Oxygen uptake was measured by the Warburg technique. At the end of the incubation period, aliquots of the medium were rapidly pipetted into trichloroacetic acid for the determinations of glucose and lactic acid, or ammonia and glutamine. The methods for these chemical analyses were also described previously (TAKAGAKI *et al.*, 1957; TAKAGAKI and TSUKADA, 1957).

For the determinations of potassium ions or amino acids in brain slices, the slices after incubation were collected, lightly drained and put into concentrated nitric acid or into 75% aqueous ethanol, respectively. The medium was poured on to a sintered glass filter to remove disintegrated slices and the filtrate used for the determinations of potassium, or glutamate (only when it was added in the medium), so that the following correction could be made (see, *Calculation and presentation of results*).

Potassium ion concentration was determined using the Shimadzu Spectrophotometer Type QR-50 with the oxygen-hydrogen flame attachment. The slices were digested with 1 ml of concentrated nitric acid in a boiling water bath for 1 hr. The digest was diluted with water and assayed for potassium. The standard potassium chloride solutions contained approximately the same concentrations of sodium and nitric acid as the unknowns.

Glutamate and other amino acids were estimated according to the method of AWAPARA (1948) and of YEMM and COCKING (1955) using a paper-chromatographic technique (Toyo Roshi filter paper no. 51). After drying the developed chromatogram the locations of amino acids were revealed by spraying lightly with 0·4% ninhydrin solution and heating at 60° for 10 sec. To insure the removal of free ammonia from the filter paper, the chromatogram was then sprayed with KOH and dried by a strong current of warm air. The spots were cut out and eluted with sodium citrate buffer of pH 5·0. The eluate was treated as described by YEMM and COCKING (1955) and estimated by photometry.

Calculations and presentations of results. Afterincudation for 60 min, the slices increased in weight. This increase was assumed (TERNER *et al.*, 1950) to be due to adherence of the medium to the slices. The increase in wet weight which occurred during incubation was determined in each type of incubation. Because the concentration of potassium or glutamate in the medium was also determined, a correction for the potassium or glutamate content of the incubated slices was applied based on the above assumption. Though PAPPIUS and ELLIOTT (1956*a*) have shown that this assumption is ont entirely justified, the corrected figures would probably approximate the true concentrations in the tissue more closely than the uncorrected ones.

All the determinations were expressed as μmole/g of initial wet weight of slices. The figures presented in the tables are means ± standard deviations. The number of experiments is given in parentheses.

RESULTS

Effect of D-*glutamate on ammonia formation and endogenous respiration in brain slices* (Table 1)

The amount of ammonia, formed by brain slices when incubated aerobically in the absence of glucose, was considerably decreased by the addition of D-glutamate (10 mM) without an increase of glutamine synthesis, although KREBS (1935) and WEIL-MALHERBE and GREEN (1955) did not find an inhibition of ammonia formation in brain by the D-isomer. D-Glutamate inhibited not only the endogenous respiration, but also the oxygen uptake of brain slices in the presence of L-glutamate as a substrate.

Free amino acids in incubated brain slices (Tables 2 and 3)

Glutamic acid occurred in brain slices in highest concentration among amino acids. In the present work, the amounts of aspartic acid and γ-aminobutyric acid as well as glutamic acid was estimated. Although the present method of quantitative

TABLE 1.—EFFECT OF D-GLUTAMATE ON AMMONIA FORMATION, ENDOGENOUS RESPIRATION AND L-GLUTAMATE OXIDATION IN BRAIN SLICES

Addition	Ammonia (μmole/g)	Glutamine (μmole/g)	Oxygen uptake (μmole/g)
No addition	8·56 ± 1·08 (13)*	0·20 ± 0·37 (13)	41·0 ± 4·0 (5)
D-Glutamate (10 mM)	2·32 ± 1·19 (5)	0·79 ± 0·23 (5)	20·2 ± 1·6 (6)
L-Glutamate (5 mM)	1·25 ± 1·12 (8)†	8·50 ± 1·91 (8)†	63·9 ± 6·3 (4)
L-Glutamate (5 mM) + D-Glutamate (10 mM)			49·7 ± 3·8 (4)

Guinea pig brain cortex slices (about 100 mg in wet weight) were incubated in 2 ml Krebs–Ringer phosphate solution of pH 7·0 without glucose at 37°C under pure oxygen. After 60 min incubation, ammonia and glutamine content in the medium was estimated. Amount of oxygen uptaken during the first 60 min of incubation after equilibration was given.

* Mean ± S.D.; figures in parentheses are number of experiments.

† Concentration of L-glutamate was 10 mM when its effect on ammonia and glutamine formation was tested.

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TABLE 2.—COMPARISON BETWEEN THE EFFECTS OF L- AND D-GLUTAMATE ON THE GLUCOSE METABOLISM OF BRAIN SLICES AND THEIR RELATION TO CHANGING THE IONIC COMPOSITION OF THE MEDIUM

Medium	Oxygen uptake (μmole/g per hr)	Glucose utilization (μmole/g per hr)	Lactate formation (μmole/g per hr)
Control	67·5 ± 4·5 (8)*	18·8 ± 2·4 (8)	16·0 ± 2·6 (8)
Potassium-free	65·2 ± 4·9 (4)	23·8 ± 2·7 (4)	27·7 ± 2·9 (4)
Potassium-rich (40 mM)	111·3 ± 9·4 (4)	42·9 ± 4·5 (4)	44·0 ± 7·0 (4)
Calcium-free	89·1 ± 8·7 (3)	31·3 ± 1·4 (3)	27·5 ± 3·0 (3)
Sodium-free	55·4 ± 8·5 (4)	34·9 ± 3·9 (4)	49·9 ± 6·0 (4)
In the presence of L-*glutamate* (10 mM)			
Control	85·5 ± 8·9 (6)	27·4 ± 2·3 (6)	38·4 ± 3·4 (6)
Potassium-free	79·7 ± 4·8 (4)	21·6 ± 2·3 (4)	26·0 ± 4·4 (4)
Potassium-rich (40 mM)	140·9 ± 8·9 (4)	39·4 ± 2·9 (4)	44·3 ± 4·4 (4)
Calcium-free	100·7 ± 4·5 (4)	38·6 ± 4·9 (4)	45·5 ± 6·6 (4)
Sodium-free	107·6 ± 3·0 (4)	29·5 ± 3·0 (4)	28·2 ± 0·7 (4)
In the presence of D-*glutamate* (10 mM)			
Control	53·9 ± 6·3 (4)	33·4 ± 1·3 (4)	46·8 ± 4·3 (4)
Potassium-free	63·7 ± 1·7 (4)	23·9 ± 2·4 (4)	29·3 ± 3·8 (4)
Potassium-rich (40 mM)	102·8 ± 9·6 (4)	38·3 ± 1·2 (4)	40·7 ± 2·4 (4)
Calcium-free	60·1 ± 6·3 (4)	44·2 ± 1·4 (4)	56·3 ± 3·8 (4)
Sodium-free	93·8 ± 5·1 (4)	31·2 ± 3·7 (4)	30·9 ± 1·9 (4)

Guinea pig brain cortex slices (about 100 mg in wet weight) were incubated under pure oxygen at 37°C in 2 ml medium for 90 min including the period of equilibration. Oxygen uptake was calculated from the amount consumed during 70 min after equilibration. Glucose utilization and lactate formation were calculated from estimations after the incubation period (90 min).

* Mean ± S.D.; figures in parentheses are number of experiments.

TABLE 3.—EFFECT OF VARIOUS METABOLIC INHIBITORS ON THE GLUCOSE METABOLISM OF BRAIN SLICES IN PRESENCE OF EITHER L- OR D-GLUTAMATE

Addition (inhibitor)	Oxygen uptake (μmole/g per hr)	Glucose utilization (μmole/g per hr)	Lactate formation (μmole/g per hr)
In presence of L-*glutamate* (10 mM)			
None	85·5 ± 8·9 (6)*	27·4 ± 2·3 (6)	38·4 ± 3·4 (6)
IAA (0·05 mM)	70·0 ± 2·2 (4)	17·0 ± 1·9 (3)	20·6 ± 2·3 (3)
Fluoride (2 mM)	73·1 ± 9·8 (4)	28·5 ± 3·3 (4)	37·9 ± 5·0 (4)
Arsenite (0·05 mM)	53·3 ± 1·3 (4)	44·8 ± 1·8 (4)	70·9 ± 5·0 (4)
Azide (0·5 mM)	62·7 ± 5·3 (4)	40·8 ± 1·2 (4)	63·1 ± 3·9 (4)
Malonate (10 mM)	48·4 ± 3·9 (3)	41·4 ± 1·4 (3)	73·1 ± 6·4 (3)
2:4-DNP (0·05 mM)	130·0 ± 4·2 (4)	43·3 ± 3·2 (4)	56·9 ± 8·8 (4)
In presence of D-*glutamate* (10 mM)			
None	53·9 ± 6·3 (4)	33·4 ± 1·3 (4)	46·8 ± 4·3 (4)
IAA (0·05 mM)	46·4 ± 5·1 (4)	13·8 ± 1·3 (4)	9·3 ± 4·2 (4)
Fluoride (2 mM)	35·6 ± 1·7 (4)	21·9 ± 2·2 (4)	31·9 ± 4·6 (4)
Arsenite (0·05 mM)	36·7 ± 1·7 (4)	39·7 ± 0·5 (4)	63·5 ± 2·5 (4)
Azide (0·5 mM)	44·8 ± 5·7 (4)	41·6 ± 2·9 (4)	74·1 ± 8·8 (4)
Malonate (10 mM)	20·5 ± 1·4 (4)	39·0 ± 2·0 (4)	70·1 ± 6·1 (4)
2:4 DNP (0·05 mM)	51·6 ± 4·4 (4)	37·9 ± 1·8 (4)	55·0 ± 7·9 (4)

Incubation and determination, see legends of Table 2.
* Mean ± S.D.; figures in parentheses give number of experiments. IAA = iodoacetic acid; DNP = dinitrophenol.

paper-chromatography did not give precise values and the deviations were rather large, the figures in Tables 2 and 3 are sufficient to show the distribution of amino acids.

On the addition of L-glutamate (10 mM) to the medium, the amount of free aspartic acid in the brain slices increased significantly, while the concentration of γ-aminobutyric acid was changed only slightly. On the addition of D-glutamate, however, the aspartic acid concentration was not increased even in the presence of glucose. On omitting sodium ions from the medium, the formation of γ-aminobutyric acid from L-glutamate was very pronounced. In this case D-glutamate was also inactive.

Effect of D-*glutamate in relation to changing the ionic milieu* (Tables 4, 5, 6 and 7)

D-glutamate, when added in the control medium containing glucose, inhibited oxygen uptake, and increased glucose utilization and lactic acid formation more than L-glutamate as already shown by WEIL-MALHERBE (1938). When potassium ions were completely omitted from the medium, D- and L-glutamate were almost without effect on aerobic glycolysis, although L-glutamate was oxidized. In the presence of a high concentration (40 mM) of potassium ions, the addition of D-glutamate did not further change oxygen uptake, glucose utilization and lactic acid formation. In a calcium-free medium, the addition of D-glutamate depressed oxygen uptake, and accelerated further

TABLE 4.—ACCUMULATION OF L- AND D-GLUTAMATE IN BRAIN SLICES

Incubation condition	Glutamic acid (μmole/g)	Aspartic acid (μmole/g)	γ-Aminobutyric acid (μmole/g)	Slice-medium ratio of glutamic acid
Before incubation	4·8 ± 1·0 (5)	2·82 ± 1·46 (3)	1·61 ± 0·53 (4)	—
After incubation				
Glucose (100 mg/100 ml)	5·0 ± 1·0 (9)	1·26 ± 0·38 (8)	1·41 ± 0·49 (8)	—
L-Glutamate (10 mM)	17·2 ± 6·8 (3)	6·82 ± 1·68 (3)	2·25 ± 0·18 (3)	2·31
L-Glutamate (10 mM) + glucose (100 mg/100 ml)	43·5 ± 12·9 (7)	8·47 ± 1·23 (5)	4·75 ± 1·71 (5)	5·53
D-Glutamate (10 mM)	8·6 ± 2·4 (6)	2·18 ± 0·83 (5)	1·69 ± 0·33 (5)	0·97
D-Glutamate (10 mM) + glucose (100 mg/100 ml)	40·6 ± 10·0 (8)	1·88 ± 0·25 (7)	2·10 ± 0·39 (7)	5·06

Guinea pig brain cortex slices (about 120–150 mg in wet weight) were incubated in 3 ml Krebs–Ringer phosphate solution of pH 7·0 at 37°C under pure oxygen for 60 min. Amino acids in the brain slices were determined by quantitative paper-chromatography. Means and standard deviations of the corrected figures are presented. See the text for the method of correction in detail. Numbers of experiments are given in parentheses.

TABLE 5.—IONIC MILIEU AND GLUTAMATE ACCUMULATION IN BRAIN SLICES

Ionic milieu	Glutamic acid (μmole/g)	Aspartic acid (μmole/g)	γ-Aminobutyric acid (μmole/g)	Slice-medium ratio of glutamic acid
In the presence of L-*glutamate* (10 mM) *and glucose* (100 mg/100 ml)				
Control	43·5 ± 12·9 (7)*	8·48 ± 1·23 (5)	4·75 ± 1·71 (5)	5·53
Potassium-free	21·0 ± 4·7 (4)	4·54 ± 0·96 (4)	4·03 ± 0·87 (4)	2·06
Calcium-free	49·5 ± 8·1 (4)	7·23 ± 0·77 (4)	1·35 ± 0·47 (4)	5·72
Sodium-free	8·0 ± 2·1 (6)	4·14 ± 0·90 (6)	10·1 ± 3·34 (6)	0·78
In the presence of D-*glutamate* (10 mM) *and glucose* (100 mg/100 ml)				
Control	40·6 ± 10·0 (8)	1·88 ± 0·25 (7)	2·10 ± 0·39 (7)	5·06
Potassium-free	25·7 ± 3·2 (4)	2·33 ± 0·30 (4)	2·12 ± 0·54 (4)	2·14
Potassium-rich (70 mM)	31·1 ± 9·6 (3)	1·38 ± 0·65 (3)	1·87 ± 0·58 (3)	3·26
Calcium-free	74·7 ± 5·6 (5)	2·12 ± 0·58 (4)	1·92 ± 0·64 (4)	6·60
Sodium-free	8·0 ± 3·7 (3)	1·61 ± 0·74 (6)	2·09 ± 0·59 (6)	0·66

For the explanation, see legends of Table 4.
* Mean ± S.D.; figures in parentheses, number of experiments.

glucose utilization and lactic acid formation. Lactic acid formation, especially, was about twice as high as that in the control calcium-free medium. In a sodium-free medium, either L- or D-glutamate greatly stimulated the oxygen uptake. This was thought to be caused by increased glucose oxidation, because the amount of consumed glucose minus half the lactic acid formed was increased. These observations show the dependence of the effects of glutamate on the ionic composition of the medium.

The potassium ion content of brain slices was decreased on incubation with

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TABLE 6.—ACCUMULATION OF POTASSIUM IONS IN BRAIN SLICES

Incubation condition	Potassium ion concentration (μmole/g)
Before incubation	45·5 ± 6·8 (18)
After incubation	
No substrate	36·3 ± 3·1 (3)
Glucose (100 mg/100 ml)	54·5 ± 1·7 (4)
L-Glutamate (10 mM)	56·5 ± 3·9 (4)
D-Glutamate (10 mM)	14·7 ± 1·4 (4)
L-Glutamate (10 mM) + Glucose (100 mg/100 ml)	82·6 ± 4·6 (5)
D-Glutamate (10 mM) + Glucose (100 mg/100 ml)	65·6 ± 6·6 (5)

Guinea pig brain cortex slices (about 100 mg in wet weight) were incubated in 2 ml Krebs–Ringer phosphate solution of pH 7·0 at 37°C under pure oxygen for 60 min. Potassium concentration in the incubated slices was determined by a flame photometric method. For the method of correction applied, see the text. Means and standard deviations are presented; numbers of experiments are given in parentheses.

TABLE 7.—IONIC MILIEU AND POTASSIUM ACCUMULATION IN BRAIN SLICES

Ionic milieu	Potassium ion concentration (μmole/g)
Control	54·5 ± 1·7 (4)
Potassium-free	37·9 (39·5, 36·2)
Potassium-rich (40 mM)	100·3 (100·7, 99·9)
Calcium-free	46·9 (48·3, 45·4)
Sodium-free	16·4 (16·8, 16·0)
In the presence of L-*glutamate* (10 mM)	
Control	82·6 ± 4·6 (5)
Potassium-free	36·5 ± 3·1 (4)
Potassium-rich (40 mM)	144·3 ± 12·8 (4)
Calcium-free	65·4 ± 3·0 (4)
Sodium-free	42·4 ± 3·6 (4)
In the presence of D-*glutamate* (10 mM)	
Control	65·6 ± 6·6 (5)
Potassium-free	33·5 ± 1·9 (4)
Potassium-rich (40 mM)	140·5 ± 9·9 (4)
Calcium-free	54·4 ± 7·1 (4)
Sodium-free	33·2 ± 1·7 (4)

For the explanation, see legends of Table 6. Brain slices were incubated in the media containing glucose (100 mg/100 ml). Means and standard deviations together with number of experiments are presented. In the case of only two experiments, each figure obtained is shown in parentheses.

D-glutamate. D-Glutamate, when added together with glucose, caused the accumulation of potassium ions in brain slices, but it was much less effective than L-glutamate plus glucose. Although D-glutamate was not taken up at all by brain slices when added alone, it was accumulated to a significant extent when added together with glucose. Its concentration in the slices was as high as five times that in the medium, just as that of L-glutamate when added with glucose. In a potassium-free medium, the content of potassium ions in slices was decreased on incubation even in the presence of glucose, although the glucose metabolism was not much changed and the oxygen uptake was rather increased. Neither L- nor D-glutamate was effective in suppressing the potassium decrease during incubation. The glutamate uptake was abolished only partly, and glutamate was concentrated in the slices about twofold as compared with the medium. On incubation in the high-potassium (40 mM-KCl) medium, the potassium content in the slices was very much increased and the addition of L- or D-glutamate further accelerated potassium accumulation. Glutamate uptake was rather decreased.

On omitting calcium ions, the potassium content in the slices was not much changed from that before incubation. The accelerating effect of glutamate on potassium uptake was clearly less than in the control medium, but glutamate uptake was stimulated; D-glutamate was less effective than L-glutamate for potassium uptake, although the former was taken up much more. In a sodium-free medium, a decrease of potassium content in the slices was observed even in the presence of glucose. The addition of D-glutamate suppressed the decrease of potassium content, although it was less effective than in the normal medium. Neither L- nor D-glutamate was taken up in the sodium-free medium.

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Effect of D-*glutamate in relation to various inhibitors* (Tables 8 and 9)

The effect of inhibitors on glucose metabolism and potassium uptake in the presence of L- or D-glutamate was studied. Generally, inhibitors which effect glucose metabolism in the absence of glutamate (TAKAGAKI *et al.*, 1958) inhibited it also in its presence. In the presence of D-glutamate, inhibitors more strongly affected the oxygen uptake, because D-glutamate was itself inhibitory for glucose oxidation. Among them, malonate (10 mM) was most effective in the inhibition of oxygen uptake.

TABLE 8.—EFFECT OF INHIBITORS ON POTASSIUM ACCUMULATION IN BRAIN SLICES IN THE MEDIUM CONTAINING GLUCOSE AND L-GLUTAMATE

Inhibitor (concentration)	Potassium ion concentration (μmole/g)
2:4-Dinitrophenol (0·05 mM)	49·3 ± 5·7 (4)*
Azide (0·5 mM)	32·0 ± 2·7 (4)
Malonate (10 mM)	42·1 ± 4·0 (4)
Fluoride (2 mM)	67·8 ± 1·8 (4)
Arsenite (0·05 mM)	58·5 ± 3·1 (4)
Iodoacetate (0·05 mM)	46·4 ± 2·6 (4)

For the explanation, see legends of Table 6.
* Mean ± S.D.; figures in parentheses, number of experiments.

D-Glutamate suppressed the acceleration of oxygen uptake due to 2:4-dinitrophenol and increased further the lactic acid formation as in the case of calcium-free medium. The extra oxygen uptake caused by L-glutamate was not additive to that due to 2:4-dinitrophenol (TAKAGAKI *et al.*, 1958).

TABLE 9.—EFFECT OF INHIBITORS ON D-GLUTAMATE ACCUMULATION IN BRAIN SLICES

Inhibitor (concentration)	Glutamic acid (μmole/g)	Slice-medium ratio of glutamic acid
2:4-Dinitrophenol (0·05 mM)	44·5 (44·6, 44·3)	4·50
Azide (0·5 mM)	18·9 ± 4·1 (5)	2·14
Malonate (10 mM)	29·0 (28·5, 29·4)	2·51
Fluoride (2 mM)	31·0 (30·0, 32·0)	2·70
Arsenite (0·05 mM)	31·5 ± 5·6 (5)	3·74
Iodoacetate (0·05 mM)	28·2 ± 13·2 (5)	3·10

For the explanation, see legends of Tables 4 and 7. Brain slices were incubated in the media containing D-glutamate (10 mM) and glucose (100 mg/100 ml). Means and standard deviations together with number of experiments are presented. In the case of only two experiments, each figure obtained is shown in parentheses.

Metabolic inhibitors also generally decreased the potassium accumulation in brain slices. Azide, at a concentration of 0·5 mM, induced a leakage of potassium ions during incubation in a medium containing glucose and L-glutamate. At that concentration, azide also inhibited D-glutamate uptake almost completely. Generally speaking, potassium accumulation was more strongly inhibited than glutamate uptake. It was impossible to establish a correlation between the effects of inhibitors on glucose metabolism and on accumulation of potassium and glutamate. In particular, 2:4-dinitrophenol, at a concentration which blocks oxidative phosphorylation in brain slices (0·05 mM), abolished potassium accumulation, but did not affect glutamate uptake.

DISCUSSION

According to the observations of WEIL-MALHERBE (1936), brain slices neither deaminate not oxidize D-glutamate, although extracts of brain tissue attack actively the D-form. STERN, EGGLESTON, HEMS and KREBS (1949) suggested the conversion of the D- into the L-form of glutamate in brain slices through deamination and reamination reactions. Judging from the present observations, it may be more natural to assume that D-glutamate is not metabolized even in the presence of glucose and that it is inhibitory for the metabolism of L-glutamate in brain slices. Aspartic and γ-aminobutyric acids, especially the latter in a sodium-free medium, were formed from added L- but not from D-glutamate. The inhibitory action of D-glutamate on synthesis and breakdown of glutamine in brain slices was demonstrated by KREBS (1935).

The finding that D-glutamate strongly inhibits the endogenous respiration and also the ammonia formation, although it is at variance with the observation of WEIL-MALHERBE and GREEN (1955), is in accordance with the author's hypothesis expressed previously (TAKAGAKI *et al.*, 1957), according to which ammonia is formed from oxidation of endogenous L-glutamate in brain slices. The discrepancy between

the present authors and WEIL-MALHERBE and GREEN (1955) may be due to the difference of concentration of added D-glutamate; the latter authors used it at a concentration of 2 mM.

The similarity between the effects of increasing potassium ion concentration, omitting calcium ions and adding glutamate or 2:4-dinitrophenol, on oxygen uptake and lactic acid formation in brain slices is well known. It has also been reported that they decrease the concentration of phosphocreatine (MCILWAIN, 1952) and inhibit the incorporation of ^{32}P into various phosphorus fractions of brain slices (FINDLAY, MAGEE and ROSSITER, 1954). STRECKER (1957) presented a possible explanation of the mechanism of these effects.

The authors, however, previously found that the incorporation of ^{32}P into phosphorus fractions, especially into the 'phosphoprotein' fraction, of brain slices was increased by potassium ions, contrary to the findings of FINDLAY *et al.* (1954). A probable cause of this discrepancy was also pointed out and the inhibitory effect of omitting calcium ions or adding 2:4-dinitrophenol on ^{32}P incorporation was confirmed (TSUKADA *et al.*, 1958). Thus, it was suggested that the effect of a high-potassium medium differs in some respects from those of a calcium-free medium or of 2:4-dinitrophenol. In a high-potassium medium, the glucose utilization and lactic acid formation were not further increased by glutamate. On the contrary, in a calcium-free medium or in the presence of 2:4-dinitrophenol, they were further stimulated by glutamate. The effect of omitting calcium ions or adding 2:4-dinitrophenol was additive with that of high potassium concentration. D-glutamate depressed the increase of oxygen uptake produced by omitting calcium ions or adding 2:4-dinitrophenol, but not that produced by potassium chloride. On the other hand, neither L- nor D-glutamate increased glucose utilization and lactic acid formation in the absence of potassium ions in the medium. These observations conclusively show that the mechanism of these phenomena is not identical, and that the effect of glutamate depends on potassium ions.

The effect on glucose metabolism of omitting sodium ions from the medium is specific to brain tissue and intimately related to that of potassium (TAKAGAKI and TSUKADA, 1957). The increased glucose utilization in the sodium-free medium was not further increased either by D- or by L-glutamate. But the amount of glucose oxidatively consumed (glucose consumption minus half the lactic acid formation) and also the oxygen uptake was surprisingly increased by either of them. The finding that potassium uptake was aided by glutamate even in the absence of sodium ions would explain this increase in oxygen uptake. Potassium ions accumulated within cells might at a relatively low level increase the oxygen uptake in a sodium-free medium.

The initial potassium content of brain slices was much lower than that reported by TERNER *et al.* (1950) or PAPPIUS and ELLIOTT (1956*b*). In the present work, brain slices were cut with a moistened blade and pooled in Ringer solution for about 20 minutes before weighing. Therefore, low potassium values were probably due to leakage or swelling during this period. At any rate, the present values for potassium content were reproducible.

According to TERNER *et al.* (1950), potassium ions are taken up by brain slices in a medium containing glucose, accompanied by the equivalent amount of L-glutamate. This was confirmed in the present investigation. But the uptake of D-glutamate in

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the presence of glucose was not paralleled by the potassium accumulation. D-Glutamate was taken up in the same way as L-glutamate, but the former was less effective than the latter in accelerating potassium uptake. Glutamate uptake occurred even in a potassium-free medium. On omitting potassium ions from the medium, the potassium content was decreased on incubation. On increasing potassium concentration, potassium uptake was increased but glutamate uptake decreased. In a calcium-free medium, uptake of glutamate, especially of the D-form, was accelerated, although less potassium ions were taken up. Sodium ions were necessary for uptake of either potassium ions or glutamate.

These observations show that the mechanism of potassium accumulation in brain slices is not the same as that of glutamate uptake. Under some conditions, glutamate was taken up without potassium, and vice versa. They also show that glutamate uptake is related not only to potassium ions but also to many other ionic conditions in the medium. Furthermore, it was impossible to establish a correlation between glucose metabolism in brain slices and potassium and glutamate accumulation, from experiments in media of various ionic composition or using various metabolic inhibitors. More knowledge of the behaviour of ions other than potassium may be required before the detailed mechanism of glutamate uptake can be elucidated.

SUMMARY

(1) The effect of D-glutamate on the aerobic metabolism of guinea pig brain cortex slices was tested, and its relation to the potassium and glutamate uptake was investigated.

(2) D-Glutamate inhibited both the endogenous oxygen uptake and the ammonia formation in brain slices.

(3) Glutamate was shown to affect glucose metabolism through the action of potassium ions.

(4) When brain slices were incubated in a medium containing glucose and D-glutamate, D-glutamate was concentrated in the slices five times more than in the medium, similarly to L-glutamate accumulation. But the accumulation of potassium ions was accelerated less by D-glutamate than by L-glutamate.

(5) The ionic composition of the incubation medium greatly affected the uptake of potassium and glutamate in brain slices. Sodium ions in the medium were indispensable for potassium and glutamate accumulations. Calcium ions were shown to inhibit glutamate uptake and accelerate potassium accumulation.

(6) An unsuccessful attempt was made to correlate the metabolism of glucose in brain slices with potassium and glutamate accumulation. It was also impossible to find a correlation between the accumulation of potassium and that of glutamate.

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MICRO-ELECTROPHORESIS IN AGAR GEL OF PROTEINS OF THE CEREBROSPINAL FLUID AND CENTRAL NERVOUS SYSTEM

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IN previous studies of the protein distribution in neurological diseases by paper electrophoresis, we found an increase in various conditions in the γ-globulins of the cerebrospinal fluid (CSF). The chemical investigation of the pathological γ-globulins in these conditions, showed that they do not always have the same chemical composition. Thus we were able to show that in the CSF of some encephalitics the γ-globulins contained a high proportion of glycoproteins (JANSSENS *et al.*, 1958). In view of the importance of the γ-globulins in syndromes such as multiple sclerosis, neurosyphilis and subacute sclerosing leucoencephalitis, we sought a method that could differentiate between the different γ-globulins. Electrophoresis in agar gel was successful in separating the γ-globulins of the CSF proteins with far better results than on paper. Paper electrophoresis was carried out by the procedure of GRASSMAN and HANNIG (1950). The protein samples applied at one end of a horizontal strip migrated towards the anode. We found that although the serum proteins applied in the centre of the agar gel migrated partially towards the anode and partially towards the cathode, the results were quantitatively similar with both methods. The same technique was also applied to the proteins of the central nervous system.

METHODS

(a) *Electrophoresis of CSF proteins in agar gel.* After concentration of 2 ml of normal CSF (LOWENTHAL, KARCHER and VAN SANDE, 1957) electrophoresis was carried out on agar gel by the procedure of WIEME and RABAEYE (1957). The CSF was concentrated by ultrafiltration at 10 atm through filters from the Membranfiltergesellschaft (Göttingen). The electrophoresis (140 v, 20 μA, 30 min) was carried out on a microscope slide covered with 1·5 mm of agar gel. The agar gel was made as follows: 1 g of agar was dissolved in 100 ml of sodium barital buffer, adjusted to pH 8·3 $\mu(= 0{\cdot}05)$. After migration, the proteins were fixed for 30 min in 5% (v/v) acetic acid made up in 70% (v/v) ethanol. They were dried at 37°C and stained with a mixture of 0·5 g amido-black, 5·0 g mercuric chloride and 5 ml acetic acid made up to 100 ml. The excess stain was removed with 5% acetic acid. The different fractions were then checked by direct densitometry, using an apparatus designed by R. RESSLER in the Laboratory of Clinical Chemistry at the Institute of Tropical Medicine, Antwerp.

(b) *Electrophoresis of proteins of the central nervous system.* The soluble proteins were extracted with 0·25 M-sucrose solution (PELLIGRINO and CARAVALIOS, 1957). A brain sample was homogenized in glassware cooled to 0°C (1 ml sucrose solution for 1 g of tissue). After centrifugation (20,000 **g** for 30 min at 0° to −5°C). The total protein concentration of the final solution was approximatively 3·0 g per 100 ml and 5 to 10 μg of this solution were used for electrophoresis in agar gel.

RESULTS

Cerebrospinal fluid. The mean values with standard deviations (S.D.) found for the main protein fractions in normal CSF by electrophoresis on paper and in agar gel are given in Table 1.

The results obtained by electrophoresis in agar gel differ in some respects from those obtained on paper. It was found that:

(a) the size of the prealbumin band is often doubled;
(b) the albumin band appears the same as on paper;
(c) α-1 and α-2 globulins can not always be separated;
(d) the β-globulins are frequently divided into two fractions;
(e) the γ-globulins appear the same as on paper, in normal CSF.

TABLE 1.—DISTRIBUTION OF PROTEINS IN THE NORMAL CSF

Method	Number of cases	Pre-albumin (%)	Albumin (%)	α-Globulins (%)		β-Globulins (%)		γ-Globulins (%)
				α-1 (%)	α-2 (%)	β-1 (%)	β-2 (%)	
Paper	44	—	66·8 ± 3·4	4·6 ± 1·0	5·3 ± 1·3	15·5 ± 2·9		7·8 ± 1·6
Agar	23	6·6 ± 1·4	63·9 ± 5·2	9·7 ± 2·7		9·6 ± 1·6	4·3 ± 1·3	5·9 ± 1·8

Results are expressed as per cent of the total protein in the sample.

The method was checked by comparing the quantitative results obtained on agar gel with those found on paper, for a series of the same cases. As shown in Table 2, both methods were applied to ten different CSFs. In the pathological CSF the proteins showed far more subfractions by electrophoresis in agar gel than on paper. The γ-globulins, in particular, divided into several fractions, as also did the β-globulins to some extent. In each case, the sum of the various subfractions nevertheless agreed with the value found on paper for the same pathological fraction, so that a comparison between the two methods could be made. The value of the method of electrophoresis in agar gel is evident in cases where pathological γ-globulins are present (Table 3).

Here the γ-globulins were divided into as many as four subfractions, some of which reached relatively high values (10 to 27%). Others remained within normal limits (4 to 7%). It was of great interest to find that these abnormal γ-globulins had different mobilities. Agar electrophoresis should help in the investigation of these subfractions, if one of them should be characteristic of a particular syndrome.

Proteins of the brain. The electrophoresis of the brain proteins was carried out with several normal human brains, one case of amaurotic idiocy, three of subacute sclerosing leucoencephalitis and the brains of many animals. All the brains were removed after autopsy. More fractions were obtained with brain tissue than with CSF and serum by electrophoresis in agar gel. It is difficult to identify these various fractions, but we named them temporarily by comparison with serum electropherograms. Unfortunately haemoglobin appears among our protein fractions, blood not having been completely removed from the brain sections.

The results for the normal human brain are shown in Table 4. Often two prealbumins are present. There is relatively less albumin than in the normal CSF and the amount of α-globulins is relatively higher than in CSF. This difference is greater for human brains than for animal brains. We are under the impression that more γ-globulins are present at the level of the brain stem and medulla, than in the higher regions of the brain: the results seem to depend on the location of the brain structure.

In the central nervous system of patients with subacute sclerosing leucoencephalitis,

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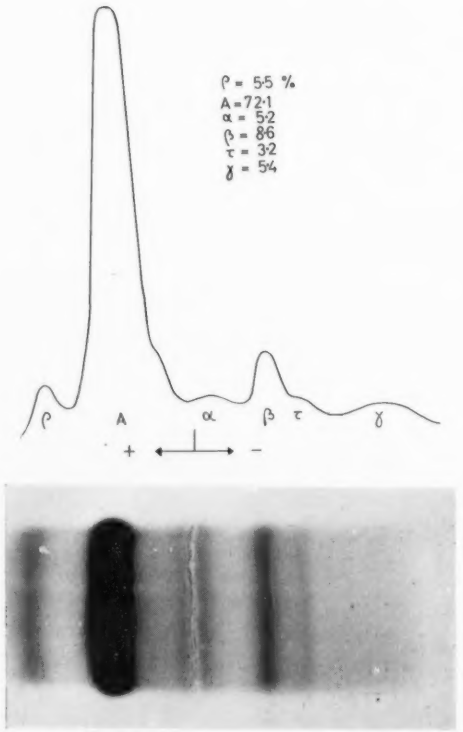

FIG 1.—Normal cerebrospinal fluid. ♂ Daw.

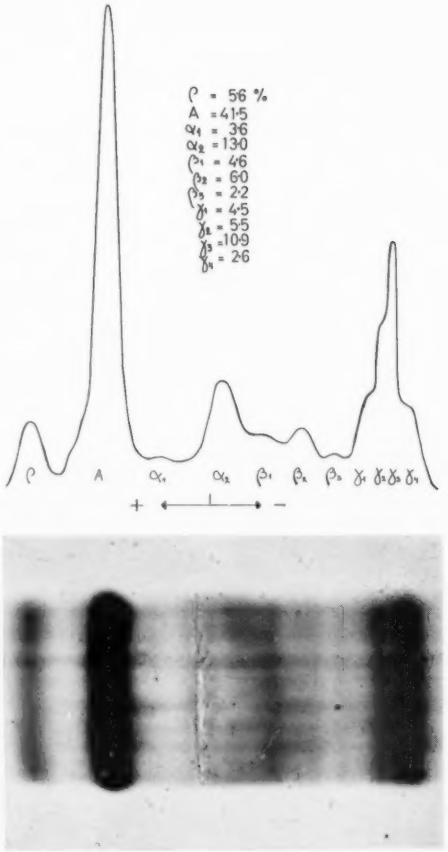

FIG. 2.—Cerebrospinal fluid of patient with subacute sclerosing leucoencephalitis. ♂ Dir.

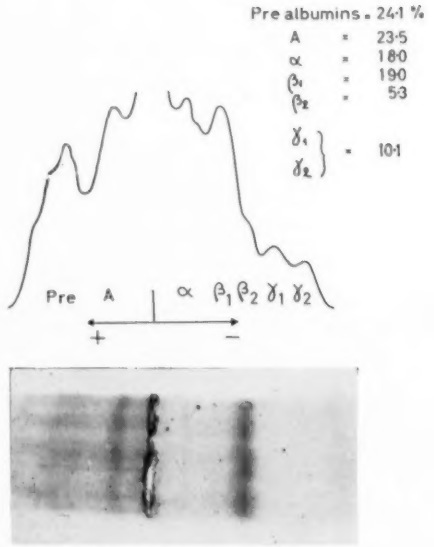

FIG. 3(a).—Normal brain tissue, white matter.

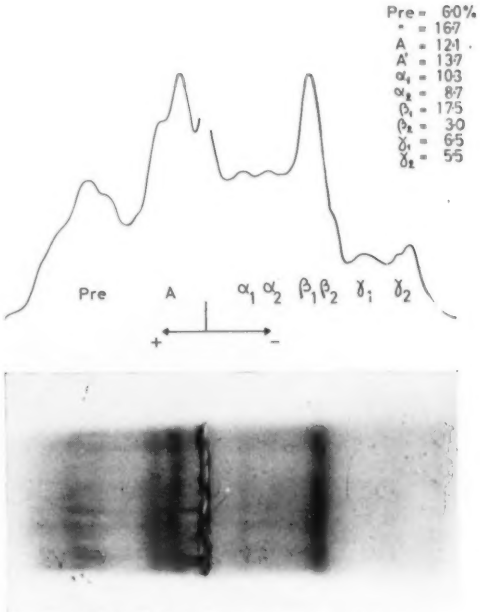

FIG. 3(b).—Normal brain tissue, brain stem.

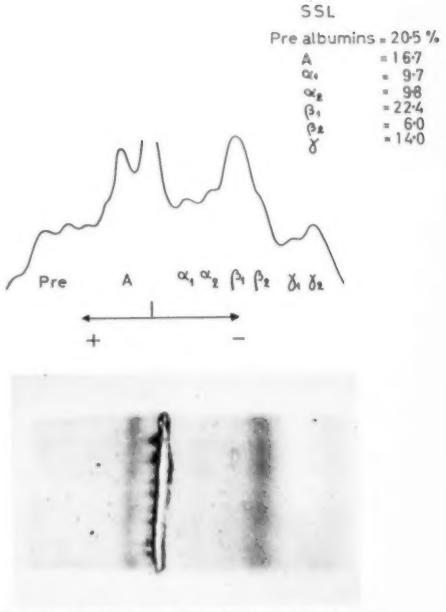

FIG. 4.—Brain tissue of patient with subacute sclerosing leucoencephalitis. White matter, ♂ Bod.

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TABLE 2.

Diagnosis	Age	Method	Pre-albumin 1 (%)	Pre-albumin 2 (%)	Albumin (%)	Globulins						
						α-1 (%)	α-2 (%)	β-1 (%)	β-2 (%)	β-3 (%)	γ-1 (%)	γ-2 (%)
Optical neuro-myelitis (Devic's Disease)	18 years	paper	—	—	64·9	6·7	4·8	14·3	—	—	9·3	—
		agar	—	7·8	59·1	6·7	7·3	9·4	4·3	—	5·4	—
Encephalitis	38 years	paper	—	—	40·4	2·8	5·3	8·2	6·2	—	37·1	—
		agar	3·1	1·7	41·8	2·4	4·7	5·3	4·2	2·4	18·2	12·3
Chorea	17 years	paper	—	3·2	57·6	6·2	6·5	16·3	—	—	10·2	—
		agar	—	6·5	71·6	7·1		8·1	3·3	—	3·4	—
Oculomotor paralysis	51 years	paper	—	—	66·5	5·6	6·7	14·5	—	—	6·7	—
		agar	—	9·4	54·9	13·3		12·7	4·0	—	5·7	—
Obstetrical encephalopathy	7 years	paper	—	—	62·9	6·5	7·1	10·0	6·6	—	6·9	—
		agar	—	6·0	58·4	10·1		11·8	5·8	—	7·9	—
Cerebral arterio-sclerosis	57 years	paper	—	—	57·5	6·0	7·5	18·6	—	—	10·4	—
		agar	—	5·9	60·1	9·3		10·0	5·5	—	9·2	—
Syringomyelia	34 years	paper	—	—	71·3	5·9	5·7	3·2	3·2	4·0	4·0	—
		agar	—	5·6	58·0	9·7		14·5	5·8	—	6·4	—
Subacute sclerosing leucoencephalitis	10 years	paper	—	—	39·1	8·4	3·9	16·5	—	—	32·1	—
		agar	—	4·7	43·6	5·9		7·1	7·0	—	4·1	27·6
Cerebral tumour*	6 years	paper	—	—	58·1	5·6	8·4	16·2	—	—	11·7	—
		agar	—	—	69·0	11·5		10·9	—	—	8·6	—
Hydrocephaly	3 months	paper	—	—	57·2	7·5	6·8	18·5	—	—	10·0	—
		agar	—	5·7	63·5	12·8		11·1	3·3	—	3·6	—

* No prior concentration of the CSF

TABLE 3.—DISTRIBUTION OF CSF PROTEINS IN FOUR CASES OF SUBACUTE SCLEROSING LEUCOENCEPHALITIS

Name	Date	Prealbumin (%)	Albumin (%)	Globulins							
				α-1 (%)	α-2 (%)	β-1 (%)	β-2 (%)	β-3 (%)	γ-1 (%)	γ-2 (%)	γ-3 (%)	γ-4 (%)
D.R.	28/6/58	5·6	41·5	3·6	13·0	4·6	6·0	2·2	4·5	5·5	10·9	2·6
	2/9/58	7·0	40·9	4·2		8·8	7·4	—	2·8	8·3	5·8	14·8
L.M.		1·6 1·5	50·6	5·5	4·0	6·1	8·0	—	4·1	27·6	—	—
T.E.		4·7	43·6	5·9		7·1	7·0	—	2·0	15·4	—	—
V.C.		5·8	51·3	5·1		12·4	—	—	7·3	11·1	7·0	—

TABLE 4.—THE DISTRIBUTION OF SOLUBLE PROTEINS IN NORMAL HUMAN BRAINS

	Age		Number of cases	Prealbumin (%)	Albumin (%)	Globulins		
						α (%)	β (%)	γ (%)
Child	3 months	grey matter	1	16·8	36·0	12·7	29·8	4·7
		white matter	1	31·8	17·8	17·8	26·9	5·7
		brain stem	1	23·1	21·8	22·4	28·1	4·5
Adults	over 18 years	grey matter	2	24·8	24·2	14·2	27·4	9·4
		white matter	4	22·4	19·5	20·8	28·4	8·9
		basal ganglia	1	9·0	17·4	32·2	34·2	2·2
		brain stem	3	25·3	27·8	13·7	21·4	11·7
		medulla	1	14·5	24·4	24·2	27·5	9·4
		cerebellum	3	25·6	20·9	23·7	23·6	6·3

we found the γ-globulins increased in comparison with corresponding areas in the normal brain; but some regions do not show this change. It is possibly due to the location of the lesions. We wish to emphasize that the γ-globulins are increased in quantity in the central nervous system in subacute sclerosing leucoencephalitis and not subdivided into several fractions as in the CSF of the same patients.

DISCUSSION

Previous studies have shown that the pathological γ-globulins in different conditions are not always the same in chemical composition (JANSSENS *et al.*, 1958). Electrophoresis in agar gel reveals in the CSF of subacute sclerosing leucoencephalitis an increase in the γ-globulins, which can be subdivided into several different fractions. These subfractions with different mobilities probably differ also in chemical composition. We never succeeded in separating them completely by paper electrophoresis, although an increase of the γ-globulins was observed. Electrophoresis in agar gel gives a better separation of the various protein fractions of the central nervous system than electrophoresis on paper. We have found that the brain proteins seem to vary in their chemical composition in different parts of the brain. Changes are

TABLE 5.—THE DISTRIBUTION OF SOLUBLE PROTEINS IN SOME PATHOLOGICAL HUMAN BRAINS

	Age (years)		Prealbumin (%)	Albumin (%)	Globulins		
					α (%)	β (%)	γ (%)
Amaurotic idiocy							
G.J.	5	cerebral hemisphere	12·3	16·8	20·0	31·9	12·0
		white matter	15·8	20·2	19·7	31·5	6·1
Leucoence-phalitis							
L.M.	13	grey matter	23·5	18·0	20·5	28·0	10·8
		anterior white matter	21·8	20·9	14·6	32·4	11·0
		white matter	19·3	24·8	13·7	30·3	12·7
		posterior white matter	11·5	25·2	17·7	35·6	10·0
		basal ganglia	21·5	15·1	12·6	41·0	9·8
		basal ganglia	20·7	14·7	29·6	27·0	8·0
		corpus callosum	19·5	30·6	22·3	19·6	8·0
		corpus callosum	19·7	30·7	17·7	21·5	10·4
B.R.	10	grey matter	14·0	20·0	21·8	33·2	11·0
		white matter	14·8	26·4	13·6	33·2	14·0
		white matter	20·5	16·7	19·5	29·3	14·0
G. W.	16	anterior white matter	26·8	22·2	22·9	14·9	8·1

TABLE 6.—DISTRIBUTION OF SOLUBLE PROTEINS IN ANIMAL BRAINS

Animal	Prealbumin (%)	Albumin (%)	Globulins		
			α (%)	β (%)	γ (%)
Monkey I (*Cercopithecus leucampyx*)	23·1	28·3	12·4	31·6	4·6
Monkey II (*Pongo pygmaeus*)	13·4	36·5	13·4	35·3	2·3
Monkey III (*Pongo pygmaeus*)	25·3	18·6	16·1	35·7	4·3
Monkey IV (*Papio papio*)	19·1	23·3	13·1	36·5	8·1
Monkey V (*Papio papio*)	20·4	22·1	24·2	27·7	5·6
Okapi (*Okapia johnstoni*)	17·5	22·4	4·4	44·8	10·9
Kangaroo I (*Macropus giganteus*)	9·3	45·0	18·1	16·6	11·0
Kangaroo II (*Macropus giganteus*)	14·3	18·0	14·3	43·5	9·9
Kangaroo III (*Marcropus giganteus*)	22·6	36·0	14·7	15·6	4·0

We have to thank the Antwerp Zoological Gardens for providing us with these brains

seen particularly in cases of subacute sclerosing leucoencephalitis, where the γ-globulins are increased in nearly every region studied; in the CSF the γ-globulins can be subdivided into several different subfractions.

SUMMARY

The proteins of the CSF and of the central nervous system can be separated by electrophoresis in agar gel. This method permits a greater separation of the γ-globulins than paper electrophoresis. The results obtained with CSF in cases of

subacute sclerosing leucoencephalitis are more informative than those obtained by paper electrophoresis.

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A METHOD FOR STUDYING METABOLIC PATHWAYS IN THE BRAIN OF THE INTACT ANIMAL

THE CONVERSION OF PROLINE TO OTHER AMINO ACIDS*

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As recently as 1957, WAELSCH wrote: 'Since the demonstration by WEIL-MALHERBE (1936) that brain cortex slices oxidized only L-glutamic acid of thirteen amino acids tested, no significant contribution has been made to the question of the oxidation of amino acids by nervous tissue'. WEIL-MALHERBE (1936) had incubated brain slices, bathed in Krebs phosphate or bicarbonate saline, with thirteen amino acids (including L-proline) and measured oxygen consumption with a Warburg manometer. Experiments such as these, conducted under conditions grossly different from those normally present in living brain, have unfortunately been interpreted by others as demonstrating that 'glutamic acid is the only amino acid utilized in the metabolism of the brain' (ADAMS and FOLEY, 1953).

GAITONDE and RICHTER (1956, 1957) injected rats intracisternally with [^{35}S]-L-methionine and found substantial labelling of cystine, glutathione, and taurine in the brain. However, as GAITONDE and RICHTER themselves point out: 'It is not known how far these changes take place in the brain and to what extent these metabolites are transported (to brain) from other organs, such as the liver'. Indeed, GAITONDE and RIGHTER present data showing that there is also intense labelling of cystine, glutathione and taurine in the liver, following an intracisternal injection of ^{35}[S]-L-methionine. There is thus evidence of copious transfer of radioactive amino acid from the brain after an intracisternal injection, and it becomes essential to control the variable of extra-cerebral metabolism of this isotope if one wishes to draw valid conclusions about cerebral pathways.

The variable of extra-cerebral metabolism can be controlled by running paired littermate experiments with one animal of a pair receiving a dose of radioactive compound *R* intracisternally and its littermate receiving the same dose intravenously. If one can show that: (1) an animal receiving a dose of *R* intracisternally has more radioactivity in other compounds isolated quantitatively from the brain than another animal receiving the same dose of *R* intravenously (since the maximum amount of *R* that could possibly leave the brain and be converted to other compounds by extra-cerebral tissues would be the entire intracisternally injected dose); and (2) intracisternal injection of *R* does not change the permeability of blood–brain barrier to *R* and its metabolites, then one may conclude that the brain itself has the ability to

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metabolize *R* to other compounds. Experiments, based on the above design, which indicate that proline is metabolized to six other amino acids in rat brain, are described in the present communication.

METHODS

Injection of rats and preparation of brain homogenates. Four male albino rats, 3 months old, of the Sprague–Dawley strain (Holtzman Co., Madison, Wis.) were used in these experiments. All injections were performed under light ether anaesthesia, using a microsyringe calibrated to 0·0001 ml. (Nuclear Science Associates, 55 Manor Drive, Newark, N.J.) All injected solutions of proline, both radioactive and inert, were 0·3 M. In experiment A, rat 1 was injected intracisternally with 32 μc of uniformly labelled [^{14}C]-L-proline in 0·01 ml of water. Rat 2, a littermate of rat 1, was injected in the exposed femoral vein with an identical radiodose of [^{14}C]-L-proline in an identical volume. In experiment B, rat 3 was injected intracisternally with 40 μc of [^{14}C]-L-proline in 0·01 ml, and 10 min later was injected in the exposed femoral vein with 0·01 ml of inert L-proline. Rat 4, a littermate of rat 3, was injected intracisternally with 0·01 ml of inert L-proline, and 10 min later was injected with 40 μc of [^{14}C]-L-proline in 0·01 ml in the exposed femoral vein. All rats were alert and walking in a co-ordinated manner within a few minutes after injections were finished.

The animals were sacrificed by decapitation 1 hr after their injection of radioisotope. Their brains were rapidly removed, cleaned of meninges, blotted with filter paper to remove any surface blood, weighed and homogenized in a Potter–Elvehjem homogenizer in 8 ml of 5% trichloroacetic acid (TCA). Additional TCA was then added to the homogenate to bring the final TCA concentration to 10%.

Preparation of acid-soluble fractions. The above homogenates were centrifuged and the clear supernatant decanted and saved. The precipitate was washed twice with 5 ml portions of 10% TCA, followed by centrifugation. The supernatants from all three centrifugations were pooled as the acid-soluble fraction. L-Ornithine·HCl (6 mg) and γ-aminobutyric acid (4 mg) were added to the acid-soluble fraction and the mixture boiled gently for 30 min in an Erlenmeyer flask. This solution was twice extracted in a separatory funnel with 25 ml portions of CCl_4, and the CCl_4 discarded. Celite Analytical Filter Aid (2–3 cm^3 of powder) was added, the solution centrifuged, and the supernatant saved. The Celite was washed twice with water and the washings added to the above supernatant. The total acid-soluble fraction was divided in half, and one part was taken down to dryness in a rotary vacuum evaporator at low temperature. A volume of 1 ml of 1 N-HCl was added to the dry residue and the resulting solution was put on a column of Dowex 50.

Preparation of protein fractions. The precipitated material left after removal of the acid-soluble fraction was washed six times with 10 ml portions of 10% TCA. The precipitate was heated for 15 min at 90° in 10% TCA to extract nucleic acids (SCHNEIDER, 1945). After centrifugation the precipitate was again washed with 10 ml of 10% TCA. The precipitate was then washed twice with 10 ml portions of acetone, refluxed for 10 min with 60 ml of $CHCl_3$–CH_3OH (1 : 1 by volume), washed three times with ether, and dried. All TCA washings and lipid extracts were discarded. The dry protein powder was then hydrolysed under reflux in 90 ml of 6 N-HCl for 24 hr. The protein hydrolysate was filtered to remove any humin, divided in half, and one part was taken down to dryness in a rotary vacuum evaporator at low temperature; 1 ml of 1 N-HCl was added to the dry residue, and the resulting solution was put on a column of Dowex 50.

Separation of amino acids. Columns (100 cm × 4 cm^2) of 200–400 mesh Dowex 50-X4, equilibrated with 1 N-HCl, were used for amino acid separations. The procedure was basically that of HIRS, MOORE and STEIN (1954), except that we did not remove aspartic acid, glutamic acid and tyrosine on Dowex 1 before putting our material on Dowex 50. Aspartic acid runs in a single peak with serine and threonine, from which aspartic acid can be totally separated by one dimensional paper chromatography, using phenol–H_2O (4 : 1) as solvent. Glutamic acid runs together with glycine, and these two amino acids are likewise easily separated on paper with phenol–H_2O. Tyrosine travels before arginine, from which it can be totally separated by proper adjustment of the gradient. In our Dowex 50 system, the γ-aminobutyric acid peak comes after the alanine–ammonia peak and before the valine peak, while the ornithine peak comes after leucine and before lysine. Because of the number of unknown components in an acid-soluble system, γ-aminobutyric acid and ornithine were further purified by paper chromatography, using both phenol–water (4 : 1) and *n*-butanol–acetic acid–water (25 : 6 : 25) as solvents.

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Determination of radioactivity. All counting was done in the Geiger region with a gas flow counter fitted with an end-window. All samples were at infinite thinness, were plated in duplicate, and were counted for no less than 1000 counts. All data reported have been corrected for background radiation.

Chemical analyses. The effluent from Dowex 50 columns was analysed for amino acids by the ninhydrin procedure of ROSEN (1957). Proline was determined by a modification (DINGMAN and SPORN, 1959) of the procedure of TROLL and LINDSLEY (1955). Nitrogen in protein hydrosates was determined by the Kjeldahl method of MILLER and MILLER (1948). Total protein in rat brain was determined by the biuret method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951).

Radioactive proline. [^{14}C]-L-Proline, uniformly labelled (Nuclear-Chicago Corp., Chicago, Ill.) was checked for radiopurity and paper chromaographic purity before injection and gave satisfactory analyses.

RESULTS

Experiment A

The distribution of radioactivity in brain protein and brain acid-soluble fractions of the four rats just prior to separation on Dowex 50 is given in Table 1. It can be seen that the total radioactivity in both the protein and acid-soluble fractions from rat 1 is more than a hundred times greater than that in the corresponding fractions of rat 2.

Table 2 gives a more detailed summary of the distribution of radioactivity in protein and acid-soluble amino acids isolated by ion-exchange and paper chromatography. The radioactivity is reported in terms of total number of counts in the quantitatively isolated amino acid. Assuming that brain protein has identical composition from rat to rat, then comparisons between rats 1 and 2, as far as the counts in any particular amino acid isolated from protein are concerned, may also be considered comparisons of specific activity. This is not true for the acid-soluble fractions, where it was necessary to add carrier γ-aminobutryic acid and ornithine. From 93 to 96 per cent of the total counts in any protein hydrolysate placed on a Dowex 50 column were recovered in the amino acid fractions isolated. Total recovery and identification of all acid-soluble counts placed on Dowex 50 columns was not attempted. Proline, glutamic acid, aspartic acid, alanine and arginine isolated from brain protein were all distinctly more radioactive in rat 1 than rat 2. All other amino acids found in brain protein were counted and found to have no measurable radioactivity. Likewise, acid-soluble γ-aminobutryic acid and ornithine were distinctly more radioactive in rat 1 than rat 2. Although experiment A demonstrates that the radioactivity in six amino acids isolated from brain is greater after intracisternal injection than after intravenous injection, one may not conclude, on the basis of this experiment alone, that brain forms these six amino acids from proline. There still exists the possibility that an intracisternal injection of proline damages the blood–brain barrier so that amino acids formed in other organs from radio-proline (that has leaked out of the brain) then might enter the brain quite readily. This possibility was controlled in experiment B.

Experiment B

The complete details of injection of rats 3 and 4 are described under Methods, and the results are summarized in Tables 1 and 2. Experiments A and B show similar results, and the data give little support to the possibility that intracisternal injection of isotonic proline increases the permeability of the blood–brain barrier with respect to proline and its metabolites. For, if intracisternal injection of proline had damaged the blood–brain barrier in this manner, then one would expect the total radioactivity found

TABLE 1.—DISTRIBUTION OF RADIOACTIVITY IN BRAIN PROTEIN AND BRAIN ACID-SOLUBLE FRACTIONS PRIOR TO SEPARATION ON DOWEX-50

Experiment	A		B	
Rat	1	2	3	4
Injection(s)	Intracisternal radio-proline	Intravenous radio-proline	Intracisternal radio-proline *followed by* intravenous inert-proline	Intracisternal inert-proline *followed by* intravenous radio-proline
Weight of animal (g)	320	310	380	370
Brain wet weight (g)	1·750	1·610	1·810	1·780
Brain total protein (mg)	131	121	136	134
Total activity of injected proline (counts/min)	20,800,000	20,800,000	25,900,000	25,900,000
Specific activity of protein (counts/min per 100 mg of brain protein)	447,000	4160	332,000	2940
Activity of acid-soluble fraction (counts/min per g wet weight brain)	2,130,000	12,500	1,990,000	15,200

in proline or its metabolites to be grossly larger in rat 4 than rat 2. The ratios(rat 1/rat 2 and rat 3/rat 4) of radioactivity in proline, glutamic acid, aspartic acid, alanine, and arginine isolated from brain proteins in experiments A and B are given in Table 3. These figures are derived from the radioactivity figures in Table 2. On the basis of our results there is good evidence that brain is capable of converting proline to glutamic acid, aspartic acid, alanine, arginine, γ-aminobutyric acid and ornithine.

DISCUSSION

The finding that brain can synthesize ornithine and arginine from proline is of particular interest. Since ammonia is exceedingly toxic to nervous tissue, the problem

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TABLE 2.—DISTRIBUTION OF RADIOACTIVITY IN AMINO ACIDS ISOLATED FROM BRAIN PROTEIN AND BRAIN ACID-SOLUBLE FRACTIONS

Experiment		A		B	
Rat		1	2	3	4
Injection(s)		Intracisternal radio-proline	Intravenous radio-proline	Intracisternal radio-proline *followed by* Intravenous inert-proline	Intracisternal inert-proline *followed by* Intravenous radio-proline
Activity of amino acids isolated from protein (counts/min per 100 mg protein)	Proline	336,000	3260	249,000	1950
	Glutamic acid	65,000	430	50,600	400
	Aspartic acid	15,100	190	13,400	160
	Alanine	1270	70	1670	110
	Arginine	410	40	400	80
Activity of amino acids isolated from acid-soluble fraction (counts/min per g wet weight brain)	γ-Amino-butyric acid	25,700	370	17,200	440
	Ornithine	470	30	1020	10

of ammonia detoxication in brain has long interested many investigators. WEIL-MALHERBE (1950), WAELSCH (1951), and STRECKER (1957) have reviewed the data which suggest that the glutamic acid–glutamine system is an important detoxifying mechanism for brain ammonia. We should like to suggest another possibility, namely that brain can also detoxify ammonia by converting ornithine to arginine, as liver is known to do in the 'ornithine cycle' (KREBS and HENSELEIT, 1932; COHEN, 1954; RATNER, 1954; BRONK and FISHER, 1956). The exact nature of the intermediates in this cycle is still a matter of some controversy, although it is fairly well accepted that ornithine plus 2 moles of ammonia plus 1 mole of carbon dioxide are converted to

arginine. The enzyme arginase, found in high concentrations in liver, then hydrolyses arginine to urea and ornithine.

In other experiments MILLER, BURKE and HAFT (1956) and BACH and SMITH (1956) have shown that glutamine is a very effective intermediate in the synthesis of urea by liver; the mechanism of action is still not certain, nor is the relationship of glutamine's enhancement of urea synthesis to the ornithine cycle known at present. It is quite possible that there are several pathways of urea synthesis, or that glutamine donates its amide nitrogen to the ornithine cycle, or that the glutamine effect may be due to relatively easy penetration of the amide into liver cells (SCHWERIN, BESSMAN and WAELSCH, 1950).

TABLE 3.—RATIOS OF RADIOACTIVITY IN AMINO ACIDS ISOLATED FROM BRAIN PROTEINS

Amino acid	Experiment A rat 1 / rat 2	Experiment B rat 3 / rat 4
Proline	103	128
Glutamic acid	151	126
Aspartic acid	80	84
Alanine	18	15
Arginine	10	5

Ratios are derived from figures in Table 2.

Inasmuch as our data indicate that the brain has some capacity to synthesize arginine, it would be of definite interest to know if brain can hydrolyse arginine to ornithine and urea. If brain possessed the ability to synthesize its own urea, this would be of definite value in detoxifying the ammonia which is believed to be produced in brain during intense central nervous system activity (RICHTER and DAWSON, 1948). We are currently conducting experiments to determine if there is arginase activity in the living brain.

Our data, which indicate that there is a highly dynamic and rapid metabolism of several amino acids in brain, contrast with the results WEIL-MALHERBE (1936) obtained with brain slices. Since many, if not almost all, neurones are ruptured in the preparation of brain slices, there may be consequent loss of metabolic co-factors and exposure of the interior of the cell to a foreign medium. Krebs phosphate and bicarbonate saline solutions used for incubation of tissue slices have molar concentrations of sodium and potassium far different from those of whole brain tissue: the sodium concentration is more than twice the normal value, while the potassium concentration is less than one-tenth of normal (MANERY, 1952). A tissue such as brain, which has become ultra-specialized in its degree of excitability, might be expected to be unusually sensitive to alterations in its normal extra- and intra-cellular ionic environment. Moreover, GEIGER *et al.* (1954, 1956) have shown that perfused brain needs factors elaborated or released by the liver to maintain normal metabolism; in the absence of such liver factors a perfused brain preparation rapidly deteriorates. These liver factors have undoubtedly been absent in most brain homogenate and slice preparations studied in

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the past. We believe that paired *in vivo* studies, of the type outlined, offer the best possibility for tracing pathways of metabolism in the living brain.

Our results also indicate that brain may well have metabolic capacities far greater than have heretofore been appreciated. On an evolutionary basis, there is no reason to suppose that brain should have lost many of the metabolic pathways that exist in primitive tissues, and one can indeed state that the greater the brain's range of metabolic activity, the greater is its capacity as a regulatory organ.

SUMMARY

Evidence that the living rat brain can convert L-proline to glutamic acid, aspartic acid, alanine, arginine, γ-aminobutyric acid and ornithine is presented. The implications of these findings with respect to: (1) previous tissue-slice experiments on amino acid oxidation in brain and (2) ammonia detoxication in brain are discussed.

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THE PENETRATION OF PROLINE AND PROLINE DERIVATIVES INTO BRAIN*

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IN studying the metabolism of a compound in the brain of an intact animal, it is important to appreciate the characteristics of that compound with respect to its penetration of the blood–brain barrier. GAITONDE and RICHTER (1956) have discussed the erroneous conclusions which may result if the blood–brain barrier is not considered in a study of metabolism of amino acids in brain. The present study was designed, therefore, as an initial experiment in an investigation of the metabolism of proline by brain (SPORN, DINGMAN and DEFALCO, 1959; DINGMAN, SPORN and DAVIES, 1959).

As DAVSON (1956) has noted, there have been few strictly quantitative studies on penetration of the blood–brain barrier by amino acids. SCHWERIN, BESSMAN and WAELSCH (1950) showed that whereas elevation of blood glutamic acid did not increase the glutamic acid concentration in brain, elevation of blood glutamine did produce a significant increase in brain glutamine. LAJTHA, FURST, GERSTEIN and WAELSCH (1957) demonstrated that while free lysine in the brain exchanges rapidly with lysine in plasma, elevation of plasma lysine does not result in corresponding elevation of brain lysine. There apparently have been no detailed studies of the penetration of blood–brain barrier by proline.

In studying the penetration of an amino acid into brain *in vivo*, one must strive to minimize the effect of brain metabolism of the amino acid. This can be done by injecting large doses of material intravenously and then analysing for changes in brain concentration immediately after injection. (Data will be presented which indicate rat brain does not metabolize proline so rapidly that a study of its penetration would be invalid if one sacrifices immediately after injection.) As DAVSON (1956) has suggested, one can prevent variations in blood levels (of the compound being studied) from skewing results by measuring the compound's penetrability as the ratio of the increase in concentration in brain over the increase in concentration in plasma (C_B/C_P). It should be pointed out that the ratio C_B/C_P, derived from measurements made immediately after injection, is not a measure of the true rate of penetration, since equilibrium is not allowed to occur (cf. ROSS, 1951; DAVSON, 1956). However, in comparing the blood–brain barrier's relative permeability toward a series of compounds, it is a useful measurement, providing that all other conditions are kept constant.

In an attempt to elucidate some of the characteristics of the proline molecule which determine its penetrability into brain, we have studied the penetration of proline,

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N-acetylproline, proline ethyl ester and *N*-acetylproline ethyl ester. We have then compared their penetrability (measured as $C_B/C_P \times 100$) with their lipid solubility and ionic charge.

MATERIALS AND METHODS

Injection of rats and preparation of brain homogenates. Male rats of the Sprague–Dawley strain (Holtzman Co., Madison, Wis.), ranging from 55 to 75 days old and from 200 to 280 g in weight, were used throughout this study. All injections were given under light ether anaesthesia into the exposed femoral vein.

Solutions to be injected were of the following molarities: *saline:* Na^+ 0·145 M, K^+ 0·005 M, Cl^- 0·150 M; *proline:* L-proline 0·3 M; *proline + ethanol:* L-proline 0·15 M, ethanol 0·15 M; *proline + saline:* L-proline 0·15 M, Na^+ 0·072 M, K^+ 0·003 M, Cl^- 0·075 M; *proline + polyethylene glycol:* L-proline 0·15 M, polyethylene glycol 0·15 M; N-*acetylproline:* *N*-acetyl-L-proline sodium salt 0·145 M, *N*-acetyl L-proline potassium salt 0·005 M (final pH, 7·5); *proline ethyl ester:* L-proline ethyl ester · H^+ 0·147 M, L-proline ethyl ester 0·003 M, Na^+ 0·030 M, K^+ 0·001 M, Cl^- 0·178 M (final pH, 6·1); N-*acetylproline ethyl ester:* *N*-acetyl-L-proline ethyl ester 0·15 M, polyethylene glycol 0·15 M. (Polyethylene glycol was 'Carbowax 400', Union Carbide Corp.)

Animals were sacrified by decapitation and their brains rapidly removed, cleaned of meninges and surface blood, weighed, and thoroughly homogenized in a Potter-Elvehjem homogenizer in 8 ml of 5% trichloroacetic acid (TCA).

Preparation of acid-soluble fraction and TCA filtrate of plasma. The homogenates were centrifuged and the supernatants decanted and saved. The precipitated material was washed twice with 5 ml portions of 5% TCA, followed by centrifugation. The supernatants from all three centrifugations were pooled as the acid-soluble fraction.

Blood from the severed neck vessels was collected in potassium oxalate bottles at the time of sacrifice. The whole blood was centrifuged, samples of the plasma treated with 5% TCA and the filtrate saved for determinations.

Proline determinations. Proline was determined by the method of TROLL and LINDSLEY (1955), modified as follows: analytical grade Amberlite IRC-50 (weak-acid cation exchange resin), buffered at pH 6·5-6·6, was used instead of Permutit; xylene was used in place of benzene; and anhydrous Na_2SO_4 was used to remove any aqueous turbidity in the xylene.

N-acetylproline and *N*-acetylproline ethyl ester gave no colour when determined directly in the above reaction. However, they can be completely determined as proline if previously hydrolysed by refluxing for 3 hr in 6 M-H_3PO_4. Proline ethyl ester gives a colour yield equivalent to 10% of its molar proline content when determined directly with the modified TROLL and LINDSLEY method, and a 100 per cent colour yield if previously hydrolysed by refluxing for 3 hr in 6 M-H_3PO_4.

Synthesis of proline derivatives. *N*-acetyl-L-proline was synthesized by the method of DU VIGNEAUD and MEYER (1932). After recrystallization from chloroform–petroleum ether, it melted at 117°–118° and gave an $[\alpha]_D^{26}$ of −114° (in aqueous solution).

L-Proline ethyl ester was synthesized according to the method of PRICE *et al.* (1950). The clear oily liquid, purified by vacuum distillation, boiled at 50°–70°/2–5 mm Hg.

N-acetyl-L-proline ethyl ester was synthesized by acetylation of L-proline ethyl ester (CHERBULIEZ and PLATTNER, 1929) and by esterification of *N*-acetyl-L-proline (MELLON *et al.*, 1953). The clear oily liquid, purified by vacuum distillation, boiled at 110°–130°/1 mm Hg, and gave an $[\alpha]_D^{28}$ of −91° (in absolute ethanol).

RESULTS AND DISCUSSION

Free proline levels in brain ½ hr after injection of proline and its derivatives

Preliminary experiments were performed to study the effect of injection of proline and its derivatives upon the level of free proline in brain. In these experiments animals were sacrificed ½ hr after completion of a rapid intravenous injection of proline or one of its derivatives. The results of this study are shown in Table 1. Of the compounds tested, proline ethyl ester alone is outstanding in its ability to elevate the level of free proline in brain. It was also found that all proline ethyl ester that had penetrated into

TABLE 1.—FREE PROLINE LEVELS IN BRAIN ONE-HALF HOUR AFTER INTRAVENOUS INJECTION OF SALINE, PROLINE AND PROLINE DERIVATIVES

Treatment	No. of rats	Free proline γ per g wet weight brain
Saline	4	28 ± 3†
Proline*	4	42 ± 5
Proline + ethanol*	2	39 ± 1
N-acetylproline*	4	26 ± 2
Proline ethyl ester*	6	200 ± 13
N-acetylproline ethyl ester*	4	27 ± 3

* Dose equivalent (on a molar basis) to 500 mg proline per kg body weight.
† Mean ± standard deviation from the mean, as calculated by SNEDECOR (1956).

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brain had been hydrolysed to free proline. A similar finding was noted when animals were sacrificed 10 min after an injection of proline ethyl ester. (The control injection of proline plus ethanol was given in order to determine whether or not ethanol formed by hydrolysis of proline ethyl ester affected the blood–brain barrier's permeability toward proline. Table 1 shows it did not.) A tentative hypothesis might be drawn from the results in Table 1, namely, that intravenous injection of an amino acid ethyl ester is a useful method for raising the brain level of a free amino acid without resorting to injection into the cerebrospinal fluid. Further study with other amino acids would be of interest.

Relative rates of penetration of proline and its derivatives into brain

The procedure for studying relative rates of penetration of proline and its derivatives into brain was as follows: (1) The isotonic solutions injected were such that both the volume of fluid and the amount of compound administered were on a body-weight basis. (2) The intravenous injections were given slowly over an 8–9 min period and the animals were sacrificed 1–2 min after the completion of the injection. (3) Both the brain acid-soluble fraction and the TCA filtrate of plasma were hydrolysed for 3 hr in 6 M-phosphoric acid prior to determination for proline. The results of these experiments are shown in Table 2. *N*-acetylproline ethyl ester was found to have the highest penetrability, followed closely by proline ethyl ester, while *N*-acetylproline and proline had very low penetrabilities. All brain acid-soluble values have been corrected for proline or proline derivative content of the blood present in brain. Figures for rats 1–16 in Table 2 for brain acid-soluble proline and plasma proline represent increases in levels over basal values found in ten saline treated animals. (These values are, respectively, 35 ± 4 μg proline per g wet weight brain and 30 ± 6 μg proline per ml plasma.) It was discovered (by analysing for proline prior to hydrolysis with 6 M-phosphoric acid) that all of the proline ethyl ester which had entered the brain had been hydrolysed by brain to free proline. Since high levels of free proline are found in brain after injection of proline ethyl ester, the low levels of free proline found in brain immediately after injection of proline itself cannot be attributed solely to rapid brain metabolism of this amino acid, and one may validly conclude that permeability to proline is low. The finding that an intravenous injection of proline does

TABLE 2.—INCREASE IN BRAIN AND PLASMA LEVELS OF PROLINE AND PROLINE DERIVATIVES AFTER INTRAVENOUS INJECTION

Compound injected*	Rat	Hydrolysed brain acid-soluble γ proline per g wet weight brain	Hydrolysed plasma filtrate γ proline per ml plasma	$C_B/C_P \times 100$
Proline + PEG	1	2	562	0·4
	2	12	508	2·4
Proline + saline	3	14	568	2·5
	4	13	622	2·1
N-Acetylproline	5	8	648	1·2
	6	12	684	1·8
	7	13	762	1·7
	8	29	793	3·7
Proline ethyl ester	9	150	230	65·3
	10	154	239	64·5
	11	135	210	64·3
	12	170	197	86·3
N-acetyl proline ethyl ester	13	330	443	74·5
	14	286	369	77·5
	15	374	470	79·6
	16	357	437	81·7

* Dose equivalent (on a molar basis) to 250 mg proline per kg body weight.

not appreciably raise the brain level of free proline is of importance in designing a study of proline metabolism in brain. Thus, if one wishes to use radio-proline to create a highly radioactive pool of free proline in brain, it can be done most easily by direct injection into the cerebrospinal fluid (e.g. intracisternally, subarachnoidally, or intraventricularly) or into the brain substance itself.

The mean $C_B/C_P \times 100$ is tabulated in Table 3 along with the lipid solubility and net charge at body pH for each of the compounds injected. Lipid solubility was determined by dissolving or suspending a known amount of each of the compounds in 15 ml of a 0·1 M-phosphate buffer, pH 7·3, and then extracting this solution successively with the following well-known lipid solvents: (a) 15 ml highly purified peanut oil, (b) 15 ml petroleum ether, and (c) 25 ml carbon tetrachloride. The amount of compound remaining in the aqueous phase was then determined. These determinations showed that 100 per cent of the proline, 91 per cent of the *N*-acetylproline, 85 per cent of the proline ethyl ester, and 50 per cent of the *N*-acetylproline ethyl ester remained in the aqueous phase. By difference, it was calculated that 0 per cent of the proline, 9 per cent of the *N*-acetylproline, 15 per cent of the proline ethyl ester, and 50 per cent

of the *N*-acetylproline ethyl ester had been extracted in the three lipid solvents. Even though the number of compounds studied in this investigation was very limited, there is evidence of a definite and positive relationship between lipid solubility and penetration into brain. Comparison of lipid solubilities and ionic charges of acetylproline and proline ethyl ester indicates that negative charge tends to inhibit penetration.

TABLE 3.—NET CHARGE, LIPID SOLUBILITY AND MEAN RELATIVE PENETRATION RATIOS OF PROLINE AND PROLINE DERIVATIVES

Compound	Net charge at body pH	Lipid solubility*	Mean C_B/C_P
Proline	⊕ ⊖	0	1·9
N-Acetyl proline	⊖	9	2·1
Proline ethyl ester	⊕	15	70·1
N-Acetyl proline ethyl ester	0	50	78·3

* Percentage of compound extracted from aqueous phase by lipid solvents (see text).

Although *N*-acetylproline ethyl ester penetrates blood–brain barrier quite readily, it is not rapidly converted to free proline by brain. This is in distinct contrast to proline ethyl ester, which brain rapidly hydrolyses. These experiments indicate that brain has greater esterase than *N*-acylase activity for the compounds tested.

SUMMARY

The penetration of blood–brain barrier by proline and *N*-acetylproline was found to be low, as compared to high penetration rates for proline ethyl ester and *N*-acetylproline ethyl ester. Intravenous injection of proline ethyl ester was also found to be effective in elevating the level of free proline within the brain. The significance of these findings with respect to the design of studies on cerebral metabolism of proline is discussed.

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THE CHEMICAL FRACTIONATION OF RAT BRAIN PROTEINS*

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ONE of the early attempts at fractionating brain proteins was made by HALLIBURTON (1894), who was able to isolate three 'proteids' by the method of fractional heat coagulation. However, MCGREGOR (1916–1917) was the first to undertake a thorough investigation of brain proteins. He experimented with the fractional precipitation of proteins using ammonium sulphate, acid and heat; and with fractional extraction of proteins using various neutral, acid, alkaline and alcoholic solvents. More recently, CHATAGNON (1951) described the separation of two protein fractions from brain, one a 'liponucleoprotein' containing both phosphorus and lipid, and the other a protein which is not associated with either phosphorus or lipid. FOLCH and LEES (1951) described the extraction and characterization (including amino acid, sulphur, phosphorus and lipid analyses) of three different proteolipid proteins from white matter. PALLADIN, POLIAKOVA and SILICH (1957) compared the incorporation of [^{35}S]-methionine by four different brain protein fractions, which were derived by successive extraction with 0·14 M-sodium chloride, 1 M-sodium chloride and 1 M-sodium hydroxide and they were able to show differences in incorporation between these fractions.

Several investigators have used electrophoretic techniques in attempting to isolate and analyse the more soluble proteins of brain. ROBERTSON (1957) was able to resolve seven distinct but grossly overlapping protein zones by paper electrophoresis of soluble rat brain proteins. Most of these investigators have named these fractions by analogy to serum proteins separated in a like manner. However, this technique does not have the advantages of a preparative method which yields sufficient material for more detailed analyses.

For these reasons we concentrated on developing a reproducible chemical method for fractionating brain proteins, whereby one could analyse each of the fractions for total protein, lipid and RNA content; percentage of proline and arginine in protein; as well as investigate incorporation of amino acid by these fractions. [^{14}C]-Proline was chosen for incorporation studies because of the importance of this amino acid in determining the stereochemistry and therefore the functional activity of protein molecules (PAULING, 1940; SZENT-GYORGYI and COHEN, 1957; YANG and DOTY, 1957).

MATERIALS AND METHODS

Isolation of protein fractions. Male albino rats of the Sprague–Dawley strain (Holtzman Co., Madison, Wis.), from 70 to 120 days old and weighing from 320 to 400 g, were used throughout this

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investigation. The procedure used for fractionating brains is the following: Animals are sacrificed by decapitation and their brains rapidly removed, cleaned of meninges and surface blood, weighed and thoroughly homogenized in a Potter–Elvehjem homogenizer at 0° in 30 ml of pH 9·5 buffer (0·04 M in Na^+ and 0·08 M in glycine). The entire fractionation procedure is carried out at from 0° to 4°.

The homogenates are then centrifuged for 30 min at 11,900 g. The supernatant from this initial centrifugation is treated as follows: 8·0 ml of saturated $(NH_4)_2SO_4$* is added slowly with constant stirring to 24 ml of the original supernatant. This mixture, which is 26 per cent saturated with respect to $(NH_4)_2SO_4$ is allowed to stand for 3–3·5 hr. It is then centrifuged and the precipitate (hereafter designated as FRACTION 1) is washed twice with 15 ml aliquots of a solution containing three parts glycine buffer plus one part of saturated $(NH_4)_2SO_4$. The washings are discarded.

Saturated $(NH_4)_2SO_4$ (4·3 ml) is added slowly with constant stirring to 28 ml of the supernatant from the precipitation of FRACTION 1. This mixture, which is 37 per cent saturated in $(NH_4)_2SO_4$, is allowed to stand overnight. It is then centrifuged and the precipitate (hereafter designated as FRACTION 2) is washed twice with 15 ml aliquots of a solution containing thirteen parts glycine buffer plus seven parts of saturated $(NH_4)_2SO_4$. The washings are discarded.

Saturated $(NH_4)_2SO_4$ (8·4 ml) is added slowly with constant stirring to 28 ml of the supernatant from the precipitation of FRACTION 2. This mixture, which is 52 per cent saturated in $(NH_4)_2SO_4$, is allowed to stand for 4–4·5 hr. It is then centrifuged and the precipitate (hereafter designated as FRACTION 3) is washed twice with 15 ml aliquots of a solution containing one part glycine buffer plus one part of saturated $(NH_4)_2SO_4$. The washings are discarded.

The supernatant from the precipitation of FRACTION 3 is taken as FRACTION 4.

The precipitate from the initial centrifugation of the original homogenate is washed four times with 30 ml of the original pH 9·5 glycine buffer, and the washings are discarded. The precipitate is then extracted with 30 ml of 1 M-NaCl by constant stirring for 30 min. The mixture is then centrifuged and the supernatant taken as FRACTION 5. The precipitate from this last centrifugation is extracted three more times with 30 ml portions of 1 M-NaCl with constant stirring for 5 min during each extraction. After each extraction the mixture is centrifuged and the supernatant added to FRACTION 5.

The precipitate remaining after the final extraction with 1 M-NaCl is extracted with 30 ml of 4 M-NaCl with constant stirring for 30 min. The mixture is allowed to stand overnight. It is then centrifuged, at which time the material partitions into three distinct layers: a floating, pale yellowish white, 'fatty' layer; a turbid fluid layer; and a precipitate. The floating layer is lifted off and placed on four layers of 120 mesh cheesecloth, which is also used to filter FRACTION 6. This floating layer is taken as FRACTION 7. The fluid layer is then decanted off through the cheesecloth filter and is taken as FRACTION 6. The remaining precipitate is extracted three more times with 30 ml portions of 4 M-NaCl with constant stirring for 5 min during each extraction. After each extraction, the mixture is centrifuged and any further floating material is added to FRACTION 7, while the filtered fluid layer is added to FRACTION 6. After the above treatment a somewhat inconstant amount of material remains which usually contains about 1 mg of protein. The latter is discarded because of difficulty in treating such a small amount of material quantitatively.

Preparation of fractions for analysis of total protein. If determinations of total protein in each of the fractions are desired, aliquots of the soluble fractions or of precipitated fractions which have been solubilized with 1 M-NaOH are precipitated with 1 vol. of 10% sodium tungstate plus 1 vol. of 0·67 N-H_2SO_4. These precipitates are then washed six times with acidified sodium tungstate and dissolved in 1 M-NaOH for biuret determinations.

Preparation of fractions for analysis of lipids, ribonucleic acid (RNA) and amino acids. Fractions are precipitated with trichloroacetic acid (TCA) at a final concentration of 20%. The TCA-precipitated material is centrifuged and the supernatant discarded. Each precipitate is washed six times with 10 ml of 10% TCA and the washings discarded. The fractions are then heated for 15 min at 90° in 10% TCA to extract nucleic acids (SCHNEIDER, 1945); these extracts are used directly for determination of RNA. After centrifugation the precipitates are again washed with 10 ml of cold 10% TCA. The fractions are then washed twice with 10 ml of acetone, refluxed for 10 min with 12 ml of $CHCl_3$–CH_3OH (1 : 1 v/v), washed three times with ether and dried. The fractions are then hydrolysed under reflux for 18–20 hr in 10 ml 6 N-HCl.

* All $(NH_4)_2SO_4$ solutions used in this procedure are saturated at room temperature.

Analytical methods. Total protein was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951). Kjeldahl nitrogen determinations were done by the method of MILLER and MILLER (1948). Arginine in protein hydrolysates was determined by the method of ROSENBERG, ENNOR and MORRISON (1956). Proline in protein hydrolysates was determined by a modification (DINGMAN and SPORN, 1959) of the method of TROLL and LINDSLEY (1955). RNA was determined by the orcinol procedure of HURLBERT, SCHMITZ, BRUM and POTTER (1954). The combined acetone, hot $CHCl_3$–CH_3OH, and ether extracts from each of the TCA-precipitated fractions were used for cholesterol and phospholipid determinations.

All radioactivity measurements were done with a gas flow counter of the Geiger type fitted with an end window. All samples were counted at infinite thinness and all data reported have been corrected for background radiation.

Methods used in [^{14}C]*-proline incorporation studies.* In carrying out studies on the effect of 8-azaguanine or electroconvulsive shock on the incorporation of [^{14}C]-proline into the various protein fractions of the rat brain the following general procedures were used: Rats under light ether anaesthesia were given intracisternal injections with a microsyringe calibrated to 0·0001 ml (Nuclear Science Associates, 55 Manor Drive, Newark, N.J.). In the 8-azaguanine study, control rats received intracisternally 0·020 ml of a solution which was 0·15 M with respect to [^{14}C]-L-proline (containing 10–20 μc) and contained 0·072 M-$NaHCO_3$ and 0·003 M-$KHCO_3$. Experimental rats received the same solution with the addition of 140 μg of 8-azaguanine. In the electroconvulsive shock study both control and experimental animals received intracisternally 0·005 ml of a solution containing 170 μg of [^{14}C]-L-proline having an activity of 10–20 μc. Experimental animals were then given electroconvulsive shocks between injection and sacrifice. The shocks (200 V for 0·1 sec) were administered with electrodes placed on the rats' ears.

[^{14}C]-L-Proline, uniformly labelled, (Nuclear-Chicago Corp., Chicago, Ill.) was checked for radio-purity and paper chromatographic purity in the following two systems: *n*-butanol–acetic acid–water and phenol–water–ammonia. 8-Azaguanine was twice recrystallized from dilute NaOH.

TABLE 1.—SEMIQUANTITATIVE ESTIMATION OF CHOLESTEROL, PHOSPHOLIPID AND RNA CONTENT OF RAT BRAIN FRACTIONS

Fraction	Cholesterol*	Phospholipid†	RNA (mg/g wet wt. brain)
1	++	0	0
2	++	0	0
3	0	0	0
4	0	0	2·8
5	+++++	+++	0·7
6	+++	+	0·6
7	+++++	++++	0

* As estimated by the Lieberman–Burchard reaction.
† As estimated by a ninhydrin test for serine and ethanolamine.

I. COMPOSITION OF FRACTIONS

General description of fractions. Mitochondria and strands of myelin are the only particles recognizable by phase microscopy which remain in the initial homogenate. The supernatant from the initial centrifugation of this homogenate contains a significant proportion of the endoplasmic reticulum, as the centrifugal force used (11,900 **g** for 30 min) is not enough to sediment this cellular component. FRACTIONS 1 through 4 represent proteins readily soluble under the conditions of low ionic strength and high pH used in homogenization. FRACTION 5 is translucent and contains mitochondria plus any cellular components solubilized in 1 M-NaCl. FRACTION 6 is also

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translucent and also contains whole and broken mitochondria with a few refractile, irregular spheres of myelin, plus any material solubilized in 4 M-NaCl. FRACTION 7 consists mainly of clumped, refractile, irregular spheres of myelin, with a small amount of entrapped mitochondria and other cytoplasmic components.

Results of analysis of fractions for lipid and nucleic acid. At this time only semiquantitative estimations of the cholesterol, phospholipid and ribonucleic acid content of each of the fractions have been made. However, as will be noted in Table 1, some separation of these components of brain tissue has been achieved using the fractionation procedure described.

Results of analysis of fractions for total protein, protein–proline, and protein–arginine. The procedure described above yields fractions which give total protein and amino acid analyses that are highly reproducible from animal to animal. The results of these analyses, which represent the mean values obtained from separate determinations made on each of six rats, are shown in Table 2. The amino acid analyses on each fraction show that there is significant degree of separation into groups of proteins with different amino acid content. However, it should be made clear that none of these fractions represents only a single protein species, but that each fraction is most likely composed of many different proteins. The proteins composing any given fraction are identical only to the extent that they behave similarly under the conditions of the fractionation procedure.

TABLE 2.—TOTAL PROTEIN, PROTEIN–PROLINE AND PROTEIN–ARGININE CONTENT OF RAT BRAIN FRACTIONS

Fraction	Total protein (mg/g wet wt. brain)	Protein–proline (% by weight)†	Protein–arginine (% by weight)†
1	3 ± 1*	5·3 ± 0·1*	7·7 ± 0·2*
2	17 ± 1	5·8 ± 0·2	8·0 ± 0·3
3	5 ± 1	4·6 ± 0·3	7·2 ± 0·8
4	11 ± 1	4·3 ± 0·3	6·4 ± 0·4
5	15 ± 1	5·1 ± 0·2	8·0 ± 0·1
6	12 ± 2	5·1 ± 0·2	7·2 ± 0·2
7	7 ± 3	4·5 ± 0·4	6·9 ± 0·4

* Mean ± standard deviation from the mean, as calculated by SNEDECOR (1956).
† All protein fractions assumed to contain 16% nitrogen.

II. EFFECT OF 8-AZAGUANINE AND ELECTROCONVULSIVE SHOCK ON INCORPORATION OF [^{14}C]-PROLINE INTO PROTEIN FRACTIONS

An important way to determine the value of a fractionation procedure for brain proteins is to investigate the functional activity of each of the fractions. The incorporation of radioactive proline into each of our fractions was studied as a first step in this direction. All injections of radioactive material were given intracisternally since we had previously found (DINGMAN and SPORN, 1959) that proline does not readily

penetrate the blood–brain barrier, and other investigators have shown that intracisternally administered radioactive amino acids are rapidly taken up by brain proteins (GAITONDE and RICHTER, 1956; CLOUET and RICHTER, 1957).

We had previously demonstrated that 8-azaguanine-2-[^{14}C], when administered intracisternally to rats, is incorporated into brain RNA; and had shown that although 8-azaguanine does not impair a rat's ability to perform a previously well-learned water-maze, it does impair the learning of a new maze (SPORN and DINGMAN, 1958). Because of these interesting results we decided to study the effect of simultaneously injected 8-azaguanine on the incorporation of [^{14}C]-proline into our protein fractions. Five pairs of littermate rats were injected intracisternally with [^{14}C]-proline while one rat from each pair was also given 8-azaguanine intracisternally. Except for one pair of littermates, which were sacrificed ½ hr after their injection, all rats were sacrificed at 1 hr. The brains of these animals were carried through the entire fractionation procedure and the protein hydrolysates analysed for radioactivity and nitrogen. The data derived from these determinations were treated as follows: the specific activity of a fraction is divided by the sum of all seven of the specific activities similarly derived for each of the fractions in any given rat. In this way any differences in the specific activity of the protein fractions, as a result of any differences in the dose of radioactive proline received by the animals, are obliterated. Only differences which are caused by differences in the incorporation of one fraction relative to the other fractions are then observed. The data are therefore reported in Table 3 as 'the relative content of radioactivity in each protein fraction'.

TABLE 3.—RELATIVE CONTENT OF RADIOACTIVITY IN EACH PROTEIN FRACTION

Fraction	Controls		8-Azaguanine		ECS	
					2 treatments	5 treatments
	½ hr (1)	1 hr (7)	½ hr (1)	1 hr (4)	1 hr (1)	1 hr (2)
1	27	26	24	26	31	26
2	21	19	21	19	19	20
3	16	16	19	16	16	17
4	13	15	13	15	12	15
5	10	12	11	12	11	12
6	6	6	6	6	5	6
7	6	5	5	7	5	5

Figures in parentheses are the number of rats in each group.

The metabolic mechanism of action of electroconvulsive therapy is at present unknown. We decided, therefore, to conduct a preliminary study on the effect of electroconvulsive shock upon the incorporation of [^{14}C]-proline into rat brain protein fractions. Accordingly, one pair of littermate rats was injected intracisternally with [^{14}C]-proline, and one rat from this pair was then given two electroconvulsive shocks, one 20 min and the other 40 min after injection. Both rats were then sacrificed 1 hr after injection. In another experiment two more pairs of littermate rats were given

intracisternal [^{14}C]-proline, and one rat from each pair was given five electroconvulsive shocks, one each at 15 min, 25 min, 35 min, 45 min and 55 min after injection. All rats were then sacrificed 1 hr after their injection. Every shock given resulted in a complete *grand mal* seizure. The results from these electroconvulsive shock experiments are also given in Table 3.

Table 3 shows that there are no significant differences between control and experimental animals. It is noteworthy, however, that there are consistent and marked differences, in the content of radioactivity, between the fractions themselves. FRACTION 1 for instance, has approximately five times the specific activity of FRACTION 7, which indicates a definite metabolic difference between the various protein fractions. We cannot say, however, that FRACTION 1 has five times the turnover or even a greater turnover than FRACTION 7, since we have no knowledge of the quantity and specific activity of the free amino acid present in the complex cellular and sub-cellular compartments from which these fractions obtain their amino acid for incorporation into protein (REINER, 1953; RUSSELL, 1958). The fact that the relative amount of [^{14}C]-labelling in the seven fractions does not change significantly between $\frac{1}{2}$ hr and 1 hr indicates only that the parameters controlling amino acid incorporation have remained isomorphic over this time period.

DISCUSSION

The use of chemical methods, of the type described in this paper, combined with more sensitive techniques for protein fractionation, such as electrophoresis, sedimentation and column chromatography (PETERSON and SOBER, 1956; TISELIUS, HJERTEN and LEVIN, 1956) should yield much new knowledge concerning the chemistry of brain proteins. These latter methods should prove more useful when the number of components being separated had first been substantially reduced. The application of fractionation techniques to brains taken from animals under a variety of experimental conditions presents an excellent opportunity to learn something of the functional importance of the fractions so isolated. Moreover, separation (by differential ultracentrifugation or density gradient techniques) of the particulate components (e.g. nuclei, mitochondria and microsomes) of nervous tissue, followed by chemical fractionation of these components, offers another possibility for improving and expanding on the technique described in this paper. Still another approach that might be used prior to chemical fractionation is some definite separation of brain into grey and white matter, or into neurones and glia.

It is likely that more sophisticated and sensitive methods of fractionation, yielding many more and purer fractions, will be devised for brain. The negative data obtained in our studies on the effect of 8-azaguanine and electroconvulsive shock do not rule out the possibility that these treatments do affect the incorporation of amino acid into brain protein. It would thus be of interest to repeat the above experiments using a more sensitive fractionation method.

SUMMARY

A chemical technique for fractionating brain proteins of rats is described. Seven different fractions were obtained which were then analysed for phospholipid, cholesterol, RNA, total protein, protein–proline and protein–arginine. The protein fractions were also analysed for radioactivity after the intracisternal injection of [^{14}C]-proline

under (a) control conditions, (b) after an intracisternal injection of 8-azaguanine, and (c) after electroconvulsive shock treatment. No differences in the incorporation of [^{14}C]-proline were noted between control and experimental animals.

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EFFECTS OF CHRONIC ADMINISTRATION OF METHAMPHETAMINE IN ENZYMIC PATTERNS IN BRAIN TISSUE

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INTRODUCTION

METHAMPHETAMINE addiction frequently induces a psychosis of which the commonest features are a paranoid-hallucinatory state without disturbance of consciousness. This man-made psychosis was seen on a large scale in Japan during the post-war period (TATETSU, GOTO and FUJIWARA, 1956; SANO and NAGASAKA, 1956; HAYASHI, 1957; BONHOFF and LEWRENZ, 1954). There are marked differences between the methamphetamine psychosis and other types of drug psychoses, such as those induced by LSD-25 and mescaline. Thus it develops in the chronic stage of intoxication and not in the acute stage, as do other model psychoses. Moreover, it persists for over a month after methamphetamine has been eliminated from the body. It seems therefore that the development of the psychosis might depend on an indirect action of the drug in causing a change in the metabolism of the brain.

These considerations led the authors to study the effects of chronic administration of methamphetamine on some of the enzymes present in brain tissue. At the start of the investigation the authors had the opportunity of investigating biopsy specimens taken from the brains of three chronic methamphetamine psychotics who were subjected to lobotomy. The psychoses had persisted in these patients for over a year after admission to hospital and they could hardly be differentiated clinically from endogenous schizophrenia. Experiments carried out *in vitro* revealed that the rate of aerobic glycolysis in brain tissue from these patients was significantly lower than that found in brain tissue from control psychopathic cases, whereas the respiratory rate was the same in both. This altered pattern of tissue metabolism has also been observed in chronic cases of schizophrenia (UTENA and EZOE, 1951).

In the present work, evidence is presented that successive administrations of methamphetamine to guinea pigs over a long period produced a significant decrease in aerobic glycolysis in brain tissue examined *in vitro* and this effect was not produced by other kinds of phenylalkylamine and psychotomimetic drugs. It was shown that methamphetamine administration produced alterations of long duration in a number of other enzyme systems.

METHODS AND MATERIALS

Adult male guinea pigs, each weighing about 500 g, were used throughout. Experiments were carried out on animals in four groups, i.e. normal control, acute experimental, and chronic experimental groups *A* and *B*. The animals of the acute experimental group received 20 mg of methamphetamine hydrochloride per kg body weight by the subcutaneous route and were killed 40 min

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later; those in the chronic experimental group received 6 mg/kg daily for periods of from 20 to 70 days. The animals of group *A*, which had received in total 60–210 mg of the drug, were killed 50 hr after the last injection; those of group *B*, which had received about 110 mg, were kept free from drug administration and examined on the fifteenth or forty-fifth day after the last injection.

In the experiments on respiration and aerobic glycolysis in brain tissue, the animals were killed by decapitation and the brain cortex was separated and prepared as slices and homogenates in the conventional way. The slices respired in oxygen and homogenates in air, and both were suspended in phosphate-glucose saline without calcium. The medium used contained 126 mM-NaCl, 5 mM-KCl, 1·2 mM-$MgSO_4$, 18 mM-Na phosphate buffer, pH 7·4, and 12 mM-glucose as substrate. The amount of tissue added per Warburg vessel was about 50 mg wet weight for slices and 100 mg for homogenates, in a final volume of 2 ml. Oxygen uptake was measured manometrically for 1 hr at 37·5° and lactic acid production was estimated by the method of BARKER and SUMMERSON (1941).

In the experiments on glycolytic activity, a fortified homogenate of brain cortex was used according to the methods of LE PAGE (1948) and UTTER, REINER and WOOD (1945). A 10% homogenate was prepared in 1·15 % KCl. The concentrations of cofactors were adjusted so that they were not the limiting factor in the overall reaction. Reaction mixtures contained, in a final volume of 3 ml, 20 mg of tissue homogenate, 4·5 mM-K–ATP, 8 mM-$MgCl_2$, 10 mM-KF, 20 mM-nicotinamide, 5 mM-Na pyruvate, 2 mM-K phosphate and 14·5 mM-$KHCO_3$ buffer of pH 7·4. Glucose or glucose-6-phosphate (10 mM) were used as substrates. Glycolytic activity was measured as carbon dioxide evolved during 20 min at 37·5°. The gas phase contained of 95% N_2 and 5% CO_2. Under these experimental conditions, glycolytic activity varied fairly linearly with the concentration of tissue.

For the measurement of monoamine oxidase activity, the modified method of SPINKS and BURN (1952) and BHAGVAT, BLASCHKO and RICHTER (1939) was used. The brain cortex was homogenized in 3 vol. of distilled water. The homogenate was dialysed for 1 hr in running tap water. The concentration of tissue was then adjusted to give a 20 per cent suspension in distilled water and 1 M-phosphate buffer pH 7·3 was added to make the final phosphate concentration 0·067 M. Liver tissue taken from the middle lobe was also prepared in the same way as the brain, except that the concentration of liver tissue was 10 per cent. The enzymic activity was measured by oxygen uptake in a Warburg vessel with tyramine as substrate. The reaction mixture used contained 1·0 ml of brain homogenate, 0·5 ml of 0·067 M-Na phosphate buffer pH 7·3, 0·2 ml of 0·01 M-HCN, 0·1 ml of distilled water and 0·2 ml of 0·15 M-tyramine hydrochloride which was tipped in from the side-arm after temperature equilibration. In the centre well 0·2 ml of 2 M-KOH was present. When liver homogenate was used, the volume of the constituents was changed to 0·9 ml of phosphate buffer, 0·2 ml of HCN, 0·1 ml of water and 0·2 ml of tyramine. The gas phase was oxygen and the temperature of the water-bath maintained at 37·5°.

The determination of cholinesterase activity was carried out by the method of AMMON (1933). The enzymic activity in the brain cortex (frontal lobe) and corpus striatum was measured separately. A Warburg vessel contained 2 ml of the homogenate into which 0·2 ml of 2·5 % acetylcholine hydrochloride was tipped at zero time. The gas phase contained of 95% N_2 and 5% CO_2, and the temperature was 37·5°. The enzymic activity was calculated from the carbon dioxide evolved in 30 min.

RESULTS

1. *The effect of methamphetamine on behaviour*

Shortly after administration, the animals showed intense psychomotor excitement, e.g. shivering, rapid walking to and fro, and biting the wire-netting of their cage while standing on their hind legs. This reaction reached a maximum at 40–60 min after the injection and subsided gradually during the following 2–3 hr. Chronic administration of the same drug produced no overt change in behaviour except for periods of transient excitement following the injection. Neither emaciation nor other bodily disturbances were observed, but careful observation revealed that the chronically intoxicated animals became more docile, passive and less responsive to external stimuli.

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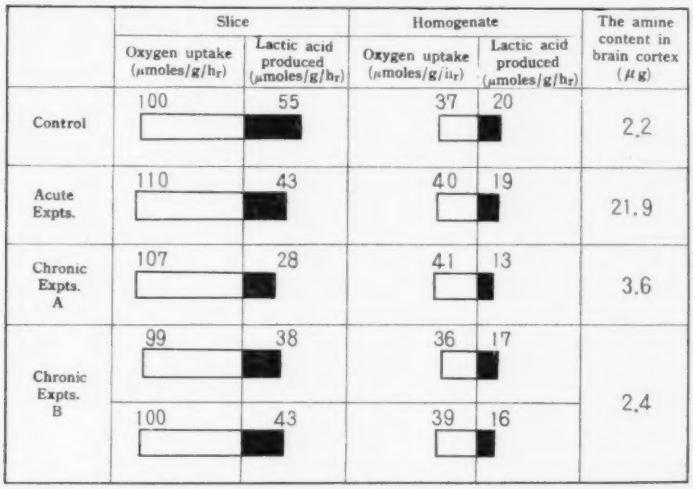

FIG. 1.—A composite chart showing the mean values of O_2 uptake and lactic acid produced in each experimental group at the administration of methamphetamine.

2. *The effects of methamphetamine on respiration and aerobic glycolysis*

The methamphetamine content of the brain and other tissues *in vivo* was determined by the methylorange method of BRODIE, UDENFRIEND, BAUER, CHENKIN and DILL (1945), and the results were given in a previous paper (UTENA, EZOE and KATO, 1955). It is relevant to mention here that the mean methamphetamine content of brain tissue was 0·15 m-moles/kg in the acute experiments, less than 0·01 m-moles/kg in the chronic group *A*, and zero in the chronic group *B* respectively.

The results of the metabolic studies on brain tissue are shown in Table 1 and Fig. 1. In the acute experiments the oxygen uptake was varied and averaged 110 per cent of the control values, while lactic acid production was slightly reduced. In the chronic

TABLE 1.—METABOLISM OF NORMAL AND METHAMPHETAMINE TREATED GUINEA PIG CEREBRAL CORTEX

Expt.	Total amount of methamphet. (mg)	Time after injection†	Oxygen uptake (μmoles/g per hr)		Lactic acid produced (μmoles/g per hr)	
			Slices	Homogenate	Slices	Homogenate
Controls			100 ± 4 (9)	37 ± 2 (7)	55 ± 9 (9)	20 ± 1 (7)
Acute expts.	10	40 min	110* ± 4 (7)	40 ± 3 (8)	43 ± 8 (6)	18 ± 6 (8)
Chronic expts. Group *A*	60–210	50 hr	107* ± 4 (7)	41 ± 4 (6)	28* ± 7 (8)	13* ± 3 (8)
Chronic expts. Group *B*	61–133	15–49 days	100 ± 5 (7)	38 ± 2 (8)	41* ± 7 (8)	17 ± 3 (8)
	61–105	15 days			39 (3)	17 (3)
	112–133	42–49 days			43 (4)	16 (4)

Means are given ± standard deviations, and in parentheses, the number of experiments.
* Indicates a statistically significant change at the $P = 0.01$ level.
† Time elapsed after the administration of methamphetamine.

experimental group *A*, the chief change found was a reduction in lactic acid production. The average decrease was 40 per cent for slices and 35 per cent for homogenates; both were statistically significant. On the other hand the increase noted in the oxygen uptake was less significant.

In the chronic experimental group *B* in which animals were, so to speak, in a convalescent state, the decrease in lactic acid production was less marked, but it was still apparent on the forty-fifth day, while the respiration values had returned to the normal range. When methamphetamine hydrochloride was added *in vitro* at concentrations of about 0·1 mM, oxygen uptake and lactic acid production were both slightly depressed (Table 1). The chronic administration of methamphetamine to guinea pigs thus caused a decrease in the aerobic glycolysis of brain tissue without impairing respiration. It appeared that these changes were not a direct effect of methamphetamine but brought about by the repeated chemical stimulation. The change was found to be reversible.

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3. *Experiments with drugs other than methamphetamine*

The observed effects of methamphetamine on the metabolism of brain tissue raise the question of how far the changes are specific for methamphetamine. To clarify this point, experiments with seven other drugs were undertaken, i.e. with three different phenylalkylamines, adrenaline, tyramine and ephedrine, and with four drugs with psychotomimetic action; mescaline, lysergic acid diethylamine (LSD-25), cocaine and diphenhydramine. All drugs were tested under acute and chronic experimental conditions.

Doses and duration of administration of these drugs were determined and are given in Table 2. In this table the effective doses for man have been given also for comparison. Procedures for animal experiments and methods for tissue studies were

TABLE 2.—DAILY DOSES AND DURATION OF ADMINISTRATION OF DRUGS

Drug	Salt	Chronic experiment		Acute experiment		Effective dose for man (mg/kg)
		Daily dose (mg/kg)	Duration (days)	Dose (mg/kg)	Time of expts.*	
Adrenaline	HCl	0·4	17–23			0·01–0·02
Ephedrine	HCl	8	22–44	80	40 min	0·4–0·8
Tyramine	HCl	6	27–52			
Mescaline	H_2SO_4	20	18–48	100–140	2–4 hr	5
LSD-25	tartrate	0·066	15–21	0·04	60 min	0·001
Diphen-hydramine	tannate	6	21–40	20	40 min	0·6
Cocaine	HCl	20	33–64	100	90 min	0·5–1

* Time elapsed after the administration of the drug.

the same as those used in the methamphetamine study. In the acute experiment, the time of decapitation of the animals was varied according to the pharmacological effect of the drug and in the chronic experiments all animals were sacrificed 50 hr after a period of daily injection.

In Fig. 2 the respiratory and glycolytic data from the methamphetamine experiments are contrasted with the results obtained with the other drugs. The results indicate that none of the drugs had so great an effect as methamphetamine. This suggested that the decrease in aerobic glycolysis in brain tissue after prolonged administration of methamphetamine was a more or less specific reaction to this drug. Ephedrine treatment produced a significant increase in aerobic glycolysis. This finding might have some connexion with the exceptionally severe histological change in nerve cells in this condition.

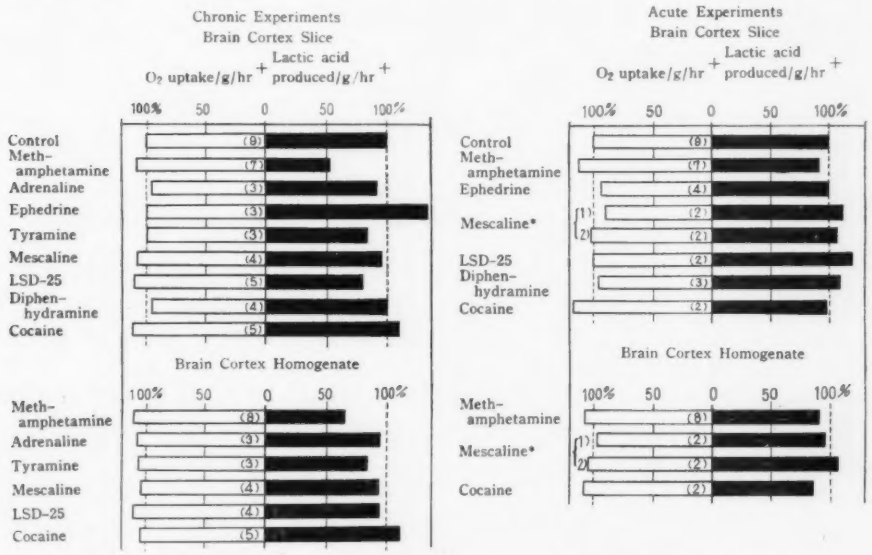

FIG. 2.—Comparison of respiratory and glycolytic activities of brain cortex in the methamphetamine and in the other drug experiments.

+ Each value expressed as per cent of control value.
* Animals were examined (1) 2 hr, (2) 4 hr after the administration of the drugs. In parentheses are given the numbers of experiments.

Injection of a large amount of mescaline (100–140 mg/kg) at one time or prolonged medication with adrenaline in a daily dose of 0·4 mg/kg for more than 20 days, gave rise to symptoms resembling catalepsy in guinea pigs. When these animals were placed flat and pressed to the floor, they retained this posture with their limbs extended and remained immobile for one or more minutes. It may be mentioned that the cataleptic symptoms could not be elicited by a single dose of adrenaline.

4. *Glycolytic activity*

In order to examine further the nature of the reduced aerobic glycolysis, glycolytic activity was measured in fortified brain homogenates under anaerobic conditions. The experiments with glucose or glucose-6-phosphate as substrate were carried out in parallel in order to elucidate the site of the disturbance in the glycolytic system.

The results of control and chronic experiments (Table 3) indicate that there was a significant decrease of about 20 per cent in glycolytic activity in the intoxicated group when glucose was used as substrate, but with glucose-6-phosphate as substrate, the

reduction was only 7 per cent and not statistically significant. These findings suggested that the disturbance was located mainly at the hexokinase step.

TABLE 3.—ANAEROBIC GLYCOLYTIC ACTIVITY IN BRAIN TISSUE

Experiment \ Substrate	Glucose	Glucose-6-phosphate
Controls	52·5 ± 2·6 (9)	55·7 ± 5·3 (7)
Chronic expts.	41·3 ± 3·5 (8)	51·7 ± 5·4 (8)
Per cent of controls	79*	93

Values are expressed as μl CO_2/20 min per 20 mg tissue.
Volume of CO_2 evolved corrected for that without substrate.
* Indicates a statistically significant change at the $P = 0{\cdot}01$ level
The number of experiments are given in parentheses.
Means are given ± standard deviation.

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5. *Monoamine oxidase activity*

Since methamphetamine is known to be a potent inhibitor of monoamine oxidase and this has been considered to be the basis of its pharmacological action, the activity of the amine oxidase in brain cortex and liver tissue was examined before (control) and after the prolonged administration of methamphetamine. The change in activity was followed at intervals from 50 hr up to 3 weeks after cessation of the daily injection. The results are shown in Table 4 and Fig. 3. At 50 hr after the last injection, the

TABLE 4.—MONOAMINE OXIDASE ACTIVITY IN THE BRAIN AND LIVER

Expts.	Total amount of amine (mg)	Time elapsed after injection	Brain (μl O_2/g per hr)	Liver (μl O_2/g per hr)
Controls		0	629 ± 15 (5) 100%	2870 ± 129 (6) 100%
Chronic expts.	132–1021	50 hr	467 ± 47 (5) 74%	2850 ± 168 (5) 100%
	156–241	1 week	738 ± 31 (5) 118%	4656 ± 286 (5) 163%
	240–444	2 weeks	850 ± 31 (3) 135%	4145 ± 232 (3) 145%
	174–200	3 weeks	745 ± 21 (3) 119%	2610 ± 46 (2) 91%

Means are given ± standard deviation.
The number of experiments are given in parentheses.

amine oxidase activity in the brain was 74 per cent of the control value, while the activity in the liver remained unchanged. But during the following 3 weeks, a remarkable increase followed by a return to normal were observed in the activity of the enzyme in both tissues. The peak values were 135 per cent in the third week in the brain and 163 per cent in the second week in the liver.

Here it must be mentioned that the methamphetamine content of the brain and liver 50 hr after the last injection was less than 0·01 m-moles/kg of tissue. Further

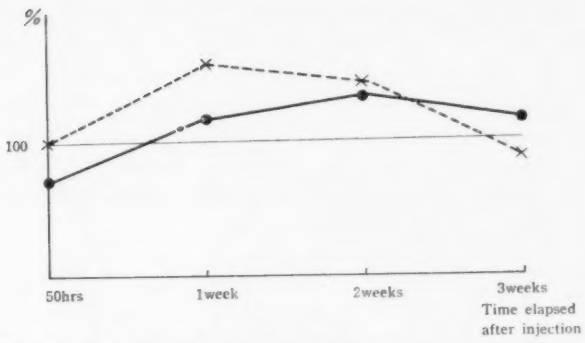

FIG. 3.—The change in monoamine oxidase activity after cessation of daily injections of methamphetamine.
× - - - × Liver tissue
● ——— ● Brain tissue

experiments with crude preparations of liver amine oxidase showed that the inhibition of enzymic activity when methamphetamine was added *in vitro* was 34 per cent at a concentration of 1·6 mM and at less than 0·16 mM no inhibition was observed. The decrease in activity in the brain was regarded therefore as an indirect effect of methamphetamine administration, and following increase as a kind of adaptive phenomenon of amine oxidase stimulation by methamphetamine.

6. *Cholinesterase activity*

Cholinesterase activity was examined in brain cortex (frontal lobe) and in the lenticular nuclei. The results shown in Table 5 indicate that the cholinesterase activity

TABLE 5.—CHOLINESTERASE ACTIVITY IN THE BRAIN TISSUE

Expts.	Total amount of amine (mg)	Brain cortex (μl CO_2/100 mg per hr)	Lenticular nuclei (μl CO_2/100 mg per hr)
Controls		487 ± 13 (4)	4860 ± 325 (4)
Chronic expts.	416–471	470 ± 16 (3)	4980 ± 122 (3)

Means are given ± standard deviation.
The number of experiments are given in parentheses.

in these parts of the brain was not influenced by the prolonged administration of methamphetamine.

DISCUSSION

It was previously shown that aerobic glycolysis in brain biopsy specimens is decreased in methamphetamine psychotics and in chronic schizophrenics (UTENA and EZOE, 1951); this finding was later confirmed by TAKAHASHI (1953). In the present paper, it is shown that in methamphetamine intoxicated animals there is a transient increase in respiration, followed by a persistent decrease in aerobic glycolysis, similar to that observed in the clinical material. It therefore appeared possible to investigate this disturbance by means of animal experiments.

In order to produce the metabolic disturbance, it is necessary to continue methamphetamine injections for more than 3 weeks. Once produced, it may last for over 6 weeks without further administration of methamphetamine, While the severity of the disturbance increases to some extent with the amount administered, the animals can withstand an astonishingly long continued medication, e.g. for 1 year, without any apparent serious consequences. Since the decrease in aerobic glycolysis can be produced only with methamphetamine, but not with other types of phenylalkylamine and psychotomimetic drugs, it is suggested that this reaction of the tissue may be more or less specific for this agent.

With regard to the histopathological findings on the methamphetamine intoxicated animals, YOKOI and AKIYAMA (1958) have observed that changes in nerve cells and glia were relatively scarce, inconstant, and unspecific when present, and were sometimes accompanied by small haemorrhages in the brain. This picture was in marked contrast with the severe changes observed in ephedrine-treated animals. On the other hand, the liver always showed spotted parenchymal degeneration, haemorrhages and fibrotic changes.

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Anaerobic glycolysis of brain homogenates fortified with cofactors was found to be significantly reduced in the chronically intoxicated animals, especially at the hexokinase step. Although the average reduction in glycolytic activity was only 20 per cent, which is far less than that observed in aerobic glycolysis, both findings could be due to a common alteration in the glycolytic system.

Alteration of monoamine oxidase activity were expected in view of the known inhibitory action of methamphetamine, but the changes observed after chronic administration were not expected. Monoamine oxidase activity was depressed slightly in the brain, while it remained unchanged in the liver. The activity in both organs increased in the following 2–3 weeks and then returned to normal. The behaviour of this enzyme suggests that the inhibitory action of the amine is related to the activation of the enzyme. In contrast with the behaviour of amine oxidase, cholinesterase activity was not influenced by drug administration. The changes observed in the amine oxidase and in the glycolytic enzymes cannot be related at present, and physiological meaning of these enzymic changes is still obscure.

SUMMARY

The chronic administration of methamphetamine to guinea pigs produce alterations in the activities of enzymes in brain tissue, which persist for a long time after cessation of the treatment. The aerobic and anaerobic *in vitro* glycolytic activity decreases significantly in this condition. These findings are similar to those observed in biopsy specimens taken from methamphetamine psychotics and chronic schizophrenics.

Changes in brain monoamine oxidase activity are also described.

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INTRACELLULAR PARTITION OF PURINE DEAMINASES IN RODENT BRAIN*

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SCHNEIDER and HOGEBOOM (1952) reported that the adenosine deaminase activity of liver homogenates prepared in an aqueous medium is localized in the non-particulate or 'soluble' fraction. Similar observations on the distributions of non-ammonia producing purine metabolizing enzymes were made by these investigators, and they suggested that the 'soluble' fraction of liver plays a role in purine metabolism. WEIL-MALHERBE and GREEN (1955) and ABOOD and GERARD (1954) described an association of adenylic acid deaminase with particulate fractions of brain and peripheral nerve. These findings raise the problem of the extent to which the intracellular distributions of purine metabolizing enzyme differ in nervous tissue and liver. To gather data pertinent to this problem, observations on the intracellular partitions in rodent brain of adenosine, guanine and guanosine deaminases were made and are reported here.

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EXPERIMENTAL

Adult male albino rabbits obtained locally and adult male rats procured from the Sprague–Dawley Company were used. Tissue was prepared and the fractions separated by procedures previously described (JORDAN and MARCH, 1956). The separation procedure is based on that of BRODY and BAIN (1952) using an aqueous 0·25 M-sucrose solution as suspension medium. Also, experiments were done in which the final supernatant of the Brody–Bain procedure was further fractionated at forces of 140,000 × gravity (g) for 1 or 1·5 hr in the Spinco ultracentrifuge (Model L), to yield a final 'soluble' fraction comparable with that obtained by SCHNEIDER and HOGEBOOM (1952) from liver. The fractions are identified as follows: centrifugal force is expressed in units equal to 1000 × g, and symbolized by *G*; time is expressed in minutes; the abbreviations sed. and sup., placed before numbers specifying the centrifugation conditions, refer respectively to the sediment or supernatant obtained with the given constants of centrifugation. Thus, sed. 0·8*G*10 refers to the sediment obtained after centrifugation at 800g for 10 min and sup. 23*G*30 to the supernatant remaining after centrifugation at 23,000g for 30 min. Sed. 0·8*G*10 consists of tissue debris, red cells, some nuclei and a few 'mitochondria'. Sed. 1·5*G*10 contains mainly nuclei with some 'mitochondria'. Sed. 23*G*30 is the 'microsome' fraction, containing many very small particles and a few 'mitochondria'. Sup. 23*G*30 usually contains a few small particles. Sup. 140*G*60 and sup. 140*G*90 are optically clear. Optical control of the fractionation procedure was carried out by phase microscopy. The fractions obtained by the Brody–Bain method were assayed in a reaction mixture composed of the following: 0·5 ml of $Na_2HPO_4 \cdot NaH_2PO_4$ buffer (1·5 M, pH 7·6), 0·25 ml of an appropriate dilution of tissue fraction, 0·5 ml of substrate solution and 0·25 ml H_2O, final volume 1·5 ml. The final concentration of guanine as substrate was $2{\cdot}11 \times 10^{-3}$ M, and with guanosine and adenosine was $2{\cdot}17 \times 10^{-3}$ M. Tissue and reagent blanks were run routinely. The reaction mixtures were incubated for 30 min at 37° with constant shaking. Ammonia was determined by the method of MUNTZ and HURWITZ (1951). In experiments on fractionation of the final Brody–Bain supernatant, the spectrophotometric method of ROUSH and NORRIS (1950) for guanase and that of KALCKAR (1947) for adenosine deaminase were employed. Nitrogen was determined by the method of MILLER and MILLER (1948).

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RESULTS

The intracellular distributions of the guanine, guanosine and adenosine deaminases are shown in Table 1. All fractions produced some ammonia from the substrates. However, the sup. 23*G*30 fraction accounted for 62 per cent of total homogenate ammonia production from guanine, 71 per cent from guanosine, and 65 per cent from adenosine. The ratios of 'specific activities' of the deaminases in this fraction to those of the homogenates were approximately three for each substrate.

When the sup. 23*G*30 fraction was subjected to additional centrifugation at 140,000 **g** for either 1 or 1·5 hr, from 96 to 98 per cent of its adenosine and guanine deaminase activities remained in the 'soluble' fraction (sup. 140*G*60 and sup. 140*G*90). There was a slight increase in 'specific activity' of the two enzymes in these fractions. Five experiments were done with each substrate. Guanosine deaminase activity was not tested.

Observations on the intracellular partitions of these enzymes in rat brain closely paralleled those on rabbit brain. Five experiments with each substrate were carried out. Additional centrifugations were not, however, done on sup. 23*G*30 from rat brain.

A brief description of experiments on partition of guanine and guanosine deaminases between one particulate (sed. 25*G*30) and its supernatant fraction (sup. 25*G*30) in mature and immature rat brain has appeared from this laboratory (JORDAN, MESSING and MARCH, 1956). Results consistent with those reported here were obtained.

In several experiments with both rabbit and rat brain, recoveries of less than 90 per cent or greater than 110 per cent were obtained. These experiments are not included in this report.

DISCUSSION

Under our experimental conditions, using an aqueous sucrose suspension medium, adenosine and guanine deaminase activities are localized predominately in the 'soluble' fraction (sup. 140*G*60 and sup. 140*G*90) of rodent brain homogenates. Guanosine deaminase activity is also predominately in the supernatant fraction (sup. 23*G*30) of the Brody–Bain fractionation procedure. These enzymes appear to differ from the adenylic acid deaminases of rat brain and peripheral nerve, which are associated with particulate matter (WEIL-MALHERBE and GREEN, 1955; ABOOD and GERARD, 1954). Very recently MENDICINO and MUNTZ (1958) reported preparation of a soluble adenosine triphosphate-activated adenylic acid deaminase from an acetone powder of dog brain. It is not clear, however, whether this enzyme would be in a 'soluble' fraction comparable to ours in brain homogenates not treated with acetone. Our findings suggest that, as in liver homogenates prepared in aqueous sucrose, the 'soluble' fraction of rodent brain homogenates plays a role in purine metabolism.

Observations on the localization of enzymes in the nuclear fraction of calf thymus and calf liver homogenates have been made which may be pertinent to our findings. STERN and MIRSKY (1953) in experiments which employed aqueous and non-aqueous suspension media reported that the adenosine deaminase of calf liver nuclei, but not of calf thymus nuclei, is washed out by an aqueous sucrose medium. It would be of importance to ascertain whether rodent brain nuclei resemble calf thymus nuclei or

TABLE 1.—DISTRIBUTION OF DEAMINASES IN DIFFERENT CELL FRACTIONS

	Adenosine			Guanine			Guanosine			Nitrogen	
Material	(μmoles NH_3 released)	(%)	('specific activity')	(μmoles NH_3 released)	(%)	('specific activity')	(μmoles NH_3 released)	(%)	('specific activity')	(mg)	(%)
Homogenate	57·5 ± 3·8		3·8	61·5 ± 3·3		4·4	35·2 ± 3·9		2·4	14·29 ± 0·45	
sed. 0·8*G*10	7·0 ± 0·8	8·5 ± 1·0	1·8	2·9 ± 0·4	4·6 ± 1·0	1·0	3·2 ± 0·4	9·9 ± 0·9	1·0	3·23 ± 0·28	22·4 ± 2·3
sed. 1·5*G*10	5·5 ± 0·8	10·1 ± 3·5	4·2	3·7 ± 0·5	5·8 ± 1·4	3·0	3·3 ± 0·9	9·4 ± 1·1	2·4	1·27 ± 0·05	8·9 ± 0·6
sed. 12*G*15	6·1 ± 0·9	9·5 ± 1·1	1·6	9·1 ± 0·9	14·5 ± 3·6	2·0	3·1 ± 0·2	8·8 ± 1·0	0·8	4·30 ± 0·18	30·3 ± 1·7
sed. 23*G*30	2·5 ± 0·4	4·1 ± 1·1	1·8	3·9 ± 0·3	6·1 ± 0·5	3·2	2·5 ± 1·8	6·9 ± 2·9	2·0	1·21 ± 0·08	8·5 ± 0·5
sup. 23*G*30	37·6 ± 2·8	65·3 ± 1·0	12·6	39·4 ± 0·9	61·9 ± 4·1	12·8	25·2 ± 3·1	71·0 ± 3·8	8·4	3·03 ± 0·24	21·1 ± 1·0
Recovery		97·5 ± 1·5			92·9 ± 2·1			106·0 ± 1·9			91·2 ± 1·2

Results represent amounts per gram of fresh tissue or its equivalent and are expressed as means and standard deviations of the means. Activity is expressed in micromoles of ammonia per hour; the 'specific activity' in micromoles of ammonia per hour per mg of nitrogen. Four experiments were done with guanine and guanosine and five with adenosine. The nitrogen values in the last column are averages from all experiments. The 'specific activities' are based on nitrogen contents of fractions assayed with each substrate.

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calf liver nuclei in this respect. If rodent brain nuclei resemble those from calf liver, then all or part of the adenosine deaminase activity (and perhaps of the guanine activity as well) which we have found in our final 'soluble' fraction, may represent enzyme washed out from the nuclear material. We have been unsuccessful, so far, in obtaining clean nuclear preparations from rodent brain with non-aqueous media to test this possiblity. Apparently it is difficult to obtain satisfactory nuclear preparations in rodent tissues with non-aqueous media, since STERN and MIRSKY report difficulties in preparing clean nuclear fractions from rat liver with these media. In this connection, it may be of significance that our fraction sed. 1·5*G*10, consisting predominately of nuclei, has a 'specific activity' equal that of the homogenate, with adenosine and guanine as substrates, although this fraction accounted for only 10 per cent of total homogenate ammonia production from these substrates. In no other fraction, except in the 'soluble' one, was such a high 'specific acitvity' encountered.

The role of purine deaminases in the physiology of the cell is not clear. Guanine, guanosine and adenosine are reported (MURRAY *et al.*, 1954) to have a slight toxic effect on tissue cultures of neoplasia derived from brain. We have been unable to find any statements of their effects on normal brain in tissue culture or *in vivo*. It has been reported (HIRSCHBERG, KREAM and GELLHORN, 1952) that neoplastic cells rich in azaguanine deaminase are more resistant to the toxic and growth inhibiting effects of azaguanine than are those poor or deficient in this enzyme. It may be that in normal brain a function of the purine deaminases is protection of the tissue from possible toxic effects of their substrates. If so, their distribution throughout the cell in the aqueous phase might expedite this function.

MCILWAIN (1957), in a recalculation of results obtained by MUNTZ (1953), states that adenylic acid is deaminated by dog brain at a rate of 60 μmoles substrate/g brain per hour. Under our conditions of assay, activities of adenosine and guanine deaminase in rabbit brain are at about this level, and guanosine deaminase is about half as active. It is perhaps worth re-emphasizing the presence of guanosine and guanine deaminase activities in brain, which was reported by WAKABAYASI (1938), since discussions of ammonia metabolism in brain frequently omit them. Thus, in two excellent recent discussions of this subject (WEIL-MALHERBE and GREEN, 1955; STRECKER, 1957), the only two purine deaminating enzymes mentioned as occurring in brain were the adenosine and adenylic acid deaminases.

SUMMARY

A 'soluble' fraction obtained from rodent brain homogenates prepared in an aqueous sucrose medium after centrifugation of the supernatant fraction yielded by the Brody-Bain separation technique at 140,000 **g** for 1 or 1·5 hours contains approximately two-thirds of the total adenosine and guanine deaminase activities of the homogenate. The 'specific' activities of these purine deaminases in the 'soluble' fraction are approximately three times those in the homogenates of rat brain. Guanosine deaminase activity is localized in the Brody–Bain supernatant fraction. It is suggested that the 'soluble' fraction of rat brain homogenates prepared in an aqueous sucrose medium plays a role in purine metabolism as it does in liver homogenates similarly treated. Attention is called to the frequent neglect of guanine and guanosine deaminases in discussions of ammonia metabolism in brain.

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PRELIMINARY NOTES

Beitrag zur Kenntnis des Stickstoffmetabolismus des Gehirns der Ratten, die auf einem akustischen epileptogenen Reiz gegenüber empfindlich oder unempfindlich sind

The Nitrogen Metabolism of the Brain in Rats that are Sensitive or Insensitive to Acoustic Epileptogene Stimulation

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In unserer früheren Arbeit studierten wir den Stickstoffstoffwechsel des Gehirns und der Leber von Ratten im praeparoxysmalen Stadium von epileptischen Anfällen (Modellanfällen), die mittels D,L-Methioninsulfoximin hervorgerufen wurden (Koloušek und Jiráček, 1959). Wir fanden, dass in diesem Stadium eine Veränderung des Stickstoffmetabolismus des Gehirns, insbesondere eine Störung des Gleichgewichtes des Zyklus: Glutaminsäure + Ammoniak ⇌ Glutamin, stattfindet. Die Funktionsaktivität des Gehirns ist nach einigen Anschauungen (Vrba, 1955, 1956) von einem bestimmten Niveau dieses Zyklus abhängig.

Die Ergebnisse unserer früheren Arbeit (Koloušek und Jiráček, 1959) führten uns zu der Annahme, dass auch der natürliche epileptische Anfall bei anfallbereiten Versuchsratten eine Folge der Störung des Zyklus: Glutaminsäure + Ammoniak ⇌ Glutamin ist. Um dies zu beweisen, haben wir Versuche mit Ratten (♂), die an unserem Institut gezüchtet und vorher in üblicher Weise sechsmal in der Schalltrommel getestet wurden, durchgeführt. Die Zeitdauer der Schalleinwirkung war 2 Minuten, die Pause zwischen den einzelnen Testen war 48 Stunden. Als Quelle des akustischen epileptogenen Reizes diente eine Schachtglocke mit einer Intensität ca. 96 Dezibell. Aus einer grossen Anzahl von Versuchsratten wurden folgende zwei Gruppen ausgewählt: (1) Ratten, die bei jedem Testversuch mit einem epileptischen Anfall auf den Reiz reagierten. (2) Ratten, bei denen im Verlaufe des Testversuchs niemals ein epileptischer Anfall hervorgerufen wurde. Lebensalter der Ratten vor dem Versuch war 8 Wochen. Vierzehn Tage vor dem eigentlichen Versuch wurden mit den Ratten keine Testversuche durchgeführt.

Zwischen dem ersten und zweiten Versuch (Zeitintervall 6 Wochen) wurden die Ratten nicht getestet. Lebensalter der Ratten, die zum ersten Versuch verwendet wurden, war dementsprechend 10 Wochen, im zweiten Versuch 16 Wochen.

Aus der Anfallbereitschaftskurve geht hervor, dass in der Periode zwischen dem dritten und vierten Monat ein Maximum der Anfallsbereitschaft liegt. Die Lebensalterdifferenz bei den Ratten im Versuch Nr. 1 und Nr. 2 wurde absichlich gewählt, um eine Veränderung der Anfallsbereitschaft auch nach der biochemischen Seite nachweisen zu können.

Die Versuchsratten wurden unter denselben Bedingungen gezüchtet und mit der Larsenschen Diät *ad libitum* gefüttert. Vor der Analysendurchführung wurden die Ratten mittels Dekapitation getötet, die Köpfe sofort in flüssigem Stickstoff gefroren und die Gehirne noch im gefrorenen Zustande herausgenommen und in flüssigem Stickstoff fein homogenisiert. In den Homogenaten der Mischproben der Gehirne wurden folgende Stickstofformen bestimmt: Gesamt-N, Eiweiss-N, Nichteiweiss-N, Ammoniak-N, Amido-N. Der Gesamtstickstoff wurde immer nach der Mineralisierung der Homogenate nach Kjeldahl mittels der Mikrodiffusionmethode (Conway, 1947) bestimmt. Der Eiweißstickstoff wurde in dem Niederschlag der Eiweißstoffe, die aus den Homogenaten mittels 20%iger Trichloressigsäure-Lösung gefällt wurden, wie der Gesamtstickstoff bestimmt. Freies Ammoniak und freie Amide wurden in den Extrakten der Homogenate, die mit 10%iger Trichloressigsäüre-Lösung bereitet wurden, nach der Methode von Richter und Dawson (1948) bzw. Harris (1943) bestimmt. Die gebundenen Amide wurden in dem nach der Extraktion zurückgebliebenen Stroma bestimmt (Vrba, *et al*: 1957, 1958).

Die Ergebnisse der Versuche sind in den Tafeln 1 und 2 verzeichnet. Zwischen den anfallsbereiten und den nicht anfallsbereiten Ratten im Lebensalter von 10 Wochen wurde eine Differenz im Gehalt des Nichteiweisstickstoffs und des Amidostickstoffs beobachtet. Bei den anfallsbereiten Ratten fand sich eine Erniedrigung des Nichteiweißstickstoffgehalts (um 19,06%) und eine Erhöhung des Amidostickstoffgehalts (um 16,3%); jedoch war die Differenz in beiden Fällen statistisch nicht signifikant (siehe Tafel 1).

TAFEL 1.—GEHALT VERSCHIEDENER STICKSTOFFORMEN IM GEHIRN DER ANFALLSBEREITEN UND NICHT ANFALLSBEREITEN RATTEN (LEBENSALTER 10 WOCHEN)

Stickstofform	%N im Trockenmaterial		Differenz im Gehalt bei anfallsbereiten und nicht anfallsbereiten (= 100%) Ratten	*P*
	Anfallsbereite Ratten	Nicht anfallsbereite Ratten		
Gesamt-N	9,07 ±0,21	10,30 ±0,45	−11,98	nicht signif.
Eiweiss-N	8,37 ±0,23	9,45 ±0,07	−11,43	nicht signif.
Nichteiweiss-N	0,69 +0,04	0,86 ±0,04	−19,77	nicht signif.
Ammoniak-N	0,0014 ±0,00005	0,0014 ±0,0001	—	—
Amido-N	0,050 ±0,004	0,043 ±0,006	+16,28	nicht signif.

Nach einer sechswöchigen Pause wurde die zweite Gruppe von Versuchsratten wie in dem ersten Versuch, verarbeitet. Statistisch gesicherte Unterschiede zwischen den anfallsbereiten und den nicht anfallsbereiten Ratten wurden bei dem Ammoniakstickstoff- und Amidstickstoffgehalt gefunden Bei den anfallsbereiten Ratten sank der Ammoniakstickstoffgehalt um 23,3%, der Amidstickstoffgehalt stieg aber um 41,7% (siehe Tafel 2).

TAFEL 2.—GEHALT VERSCHIEDENER STICKSTOFFORMEN IM GEHIRN DER ANFALLSBEREITEN UND NICHT ANFALLSBEREITEN RATTEN (LEBENSALTER 16 WOCHEN)

Stickstofform	%N im Trockenmaterial		Differenz im Gehalt bei anfallsbereiten und nicht anfallsbereiten (= 100%) Ratten	*P*
	Anfallsbereite Ratten	Nicht anfallsbereite Ratten		
Gesamt-N	9,01 ±0,21	9,00 ±0,11	+0,11	nicht signif.
Eiweiss-N	7,80 ±0,13	7,79 ±0,06	+0,12	nicht signif.
Nichteiweiss-N	1,21 ±0,002	1,21 ±0,05	—	—
Ammoniak-N	0,0023 ±0,0002	0,0030 ±0,00015	−23,34	<0,01
Amido-N	0,146 ±0,014	0,103 ±0,010	+41,74	<0,05

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Die Bestimmung des Gehalts der gebundenen Amide im Gehirn zeigt keine Unterschiede zwischen den anfallsbereiten und nicht anfallsbereiten Ratten; dies kann aber methodische Ursachen haben.

Die Ergebnisse der Versuche Nr. 1 und 2 lassen vermuten, dass während der Ontogenese eine Erhöhung der Anfallsbereitschaft stattfindet, weil im Versuch mit den älteren Ratten statistisch gesicherte Unterschiede im Gehalt an Stickstoff, an freiem Ammoniak und an freien Amiden zwischen den anfallsbereiten und nicht anfallsbereiten Ratten vorhanden waren.

Die Ergebnisse der früheren Versuche mit Methioninsulfoximin und der vorliegenden zeigen einen grundsätzlichen Unterschied im Gehalt an freien Amiden im Gehirn. Wie nach einer Injektion von Methioninsulfoximin der Gehalt der freien Amide im Gehirn im präparoxysmalen Stadium sinkt (KOLOUŠEK and JIRÁČEK, 1959), so wurde hier bei den anfallsbereiten Ratten im Gehirn im Ruhestadium ein grösserer Gehalt an freien Amiden gefunden. Bei der Bestimmung des Ammoniaks im Harn der anfallsbereiten und nicht anfallsbereiten Ratten im Ruhestadium wurden Unterschiede gefunden (KOLOUŠEK, 1958*b*). Bei den anfallsbereiten Ratten fand sich eine statistisch gesicherte Erniedrigung der Ammoniakausscheidung im 24-Stunden-Harn (KOLOUŠEK und HORÁK, 1958). 1958*b*). Dieser Befund steht im Einklang mit unserem Befund, dass anfallsbereite Ratten im Ruhestadium einen kleineren Gehalt an Ammoniak im Gehirn als nicht anfallsbereiten Ratten besitzen. Daraus würde hervorgehen, dass im Ruhestadium ein Unterschied zwischen dem Niveau des Stickstoffmetabolismus des Gehirns der anfallsbereiten und nicht anfallsbereiten Ratten existiert.

Nach der Hypothese von VRBA (1955, 1956) entspricht einem bestimmten Niveau des Zyklus: Glutaminsäure + Ammoniak ⇌ Glutamin eine bestimmte Aktivität des Nervensystems. Nach dieser Vorstellung kann die gefundene Veränderung des Niveaus dieses Zyklus bei anfallsbereiten Ratten im Ruhestadium, die sich durch eine Erhöhung des Stickstoffgehalts der freien Amide um 41,7% im Vergleich mit den nicht anfallsbereiten Ratten äussert, als eine Ursache der Veränderung der Aktivität des Gehirns angesehen werden. Diese Veränderung äussert sich dann in einem epileptischen Anfall, der durch einen entsprechenden äusseren Anlass, in unserem Falle durch einen akustischen Reiz, hervorgerufen werden kann.

SUMMARY

The nitrogen metabolism of the brain of rats which were susceptible or not susceptible to acoustic epileptiform seizures has been studied. It was found, that the brains of the rats that had seizures (age 16 weeks) contained a larger amount of free amide-N and a smaller amount of free ammonia-N than the brains of the rats that had not. The difference was statistically significant. The brains of younger rats susceptible to seizures (age 10 weeks) contained a large amount of free amide-N and a smaller amount of nonprotein-N than the brains of rats that were not susceptible, but the difference was not statistically significant.

The differences might be attributed to a disorder of the biologically important reaction: glutamic acid + ammonia ⇌ glutamine.

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Stickstoffmetabolismus des Gehirns und der Leber bei Ratten nach einer Applikation von Methioninsulfoximin.

The Nitrogen Metabolisim of the Rat Brain and Liver after Administration of Methioninesulphoxime

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METHIONINSULFOXIMIN (Abkürzung MSI), ein Abkömmling des Methionins (Abkürzung ME), ist für eine Anzahl lebender Organismen ein sehr giftiger Stoff. Die Intoxikation der Versuchstiere durch diesen Stoff, der schon in sehr kleinen Konzentrationen epileptische Anfälle hervorruft, wurde schon von mehreren Autoren studiert (GERSHOFF, 1951, 1956; LODIN und KOLOUŠEK, 1956). PACE und MCDERMOTT (1952) berichteten, dass MSI die Samenkeimung hemmt und dass die Biosynthese des Glutamins aus dem Glutamat bzw. der Glutamohydroxamsäure aus Glutamin und Hydroxylamin *in vitro* durch MSI gehemmt wird. Über die Wirkungsweise dieses Stoffes im tierischen sowie pflanzlichen Organismus ist zur Zeit noch nicht viel bekannt.

Um den Befund von PACE und MCDERMOTT (1952) auch *in vivo* nachzuprüfen, haben wir Versuche an Ratten (♂, Durchschnittsgewicht 200 Gramm), die mit einer modifizierten Larsen–Diät gefüttert wurden, durchgeführt. D,L-MSI wurde grundsätzlich nach BENTLEY *et al.*, (1951) synthetisiert und intraperitoneal in einer krampferzeugenden Dosis (400 mg/kg) injiziert. Den Kontrolltieren wurde ein entsprechendes Volumen der physiologischen Lösung intraperitoneal injiziert. Die Versuchstiere wurden 6 Stunden nach der Applikation des MSI mittels Dekapitation getötet. Die Köpfe und exstirpierten Lebern wurden sofort in flüssigem Stickstoff gefroren. Das Gehirn wurde dann noch im gefrorenen Zustande herausgenommen und in flüssigem Stickstoff fein homogenisiert; die Lebern wurden auf dieselbe Weise verarbeitet. Sechsstündiges Interval wurde teils gewählt, damit der Zustand gerade vor dem Anfall studiert werden kann (nach 6 Stunden beginnt sich der epileptische Zustand zuentwickeln), teils deshalb, weil in dieser Zeit die Konzentration des MSI im Gehirn das Maximum erreicht hat und das MSI noch nicht in die Eiweissmoleküle eingebaut ist (KOLOUŠEK and BABICKÝ, 1957).

In den Homogenaten des Gehirns und der Leber wurden folgende Stickstofformen bestimmt: Gesamt-N, Eiweiss-N, Nichteiweiss-N (aus der Differenz zwischen dem Gesamt-N und Eiweiss-N berechnet), Ammoniak-N und Amido-N. Ausserdem wurden auch freie Aminosäuren und Peptide in den Extrakten der Gehirnhomogenate studiert.

Gesamtstickstoff wurde nach der Mineralisation der Homogenate (nach KJELDAHL) mittels der Mikrodiffusionsmethode nach CONWAY (1947) bestimmt. Eiweisstickstoff wurde in den mit 20%-iger Trichloressigsäure aus den Homogenaten gefällten Eiweisstoffen, auf dieselbe Weise wie der Gesamtstickstoff, bestimmt. Freies Ammoniak wurde in Extrakten der Homogenate mit 10%iger Trichloressigsäure nach RICHTER und DAWSON (1948) bestimmt. In denselben Extrakten wurden die freien Amide nach HARRIS (1943) bestimmt. Die freien Aminosäuren und Peptide wurden aus den Gehirnhomogenaten mit siedender 0,01 N-Essigsäure bzw. mit 75% igem Äthanol in der Kälte extrahiert und dann mittels zweidimensionaler Papierchromatographie analysiert (KOLOUŠEK, 1957).

Aus den Ergebnissen der Bestimmungen, die in der Tafel 1 wiedergegeben sind, geht hervor, dass 6 Stunden nach der Injektion von D,L-MSI bedeutende Veränderungen im Stickstoffmetabolismus des Gehirns und der Leber stattfinden. Im Gehirn wurde eine Hemmung der Bildung der freien Amide beobachtet. Die Erniedrigung des Eiweisstickstoffgehaltes im Gehirn, die durch MSI hervorgerufen wird, kann als eine Folge der Hemmung der Amidbildung angesehen werden, weil die Amide eine wichtige Rolle bei der Proteinsynthese spielen. Die Hemmung der Amidbildung die von uns beobachtet wurde, steht im Einklang mit früheren *in vitro* Befunden (PACE und MCDERMOTT, 1952) und mit Versuchsergebnissen an keimenden Erbsensamen, wo MSI eine kräftige Hemmung der Asparaginbildung verursacht (KOLOUŠEK und LODIN, 1958). Die gefundene Erniedrigung des Ammoniakgehaltes im Gehirn kann als eine Folge der Hemmung der Glutaminbildung erklärt werden; dies verursacht dann eine Störung des Zyklus: Glutaminsäure + NH_3 ⇌ Glutamin (VRBA, 1955, 1956).

In der Leber konnten keine statistisch signifikanten Differenzen im Gehalt an freiem Ammoniak sowie an freien Amiden gefunden werden. Jedoch konnte eine kleine Erhöhung des Eiweisstick-

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TAFEL 1.

% N im Trockengewicht	Kontroll-Versuch	MSI	Differenz zwischen Kontroll- und MSI-Versuch in % (Kontrolle = 100%)	*n*	*P*
	Gehirn				
Gesamt-N	9,47 ±0,17	9,76 ±0,50	+3,0	10	nicht. signif.
Eiweiss-N	8,58 ±0,02	8,41 ±0,05	−2,0	10	<0,002
Nichteiweiss-N	0,89 —	1,35 —	+51,7	10	<0,002
Ammoniak-N	0,0026 ±0,0002	0,0021 ±0,0002	−19,2	10	<0,01
Amido-N (frei)	0,090 ±0,005	0,050 ±0,003	−44,4	10	<0,001
	Leber*				
Gesamt-N	10,72 ±0,13	10,89 ±0,14	+1,6	10	nicht signif.
Eiweiss-N	10,00 ±0,10	10,40 ±0,05	+4,0	10	<0,001
Nichteiweiss-N	0,72 —	0,50 —	−31,11	10	<0,001

* In der Leber wurden keine statistisch signifikanten Differenzen im Ammoniak-N- und Amido-N-Gehalt zwischen den Kontroll- und MSI-Versuchstieren beobachtet. Die Werte werden deshalb auch nicht angegeben.

stoffgehaltes beobachtet werden. Dieser Befund steht im Einklang mit der früheren Beobachtung, dass nach der Injektion des MSI die Inkorporation des ME-^{35}S im Gehirn sinkt, dagegen in der Leber steigt (KOLOUŠEK und JIRÁČEK, 1958).

Zweidimensionale Papierchromatographie zeigte keine qualitativen Veränderungen der freien Aminosäuren und Peptide im Gehirn der Tiere, die MSI erhielten, im Vergleich zu den Kontrolltieren. Eine visuelle semiquantitative Abschätzung der Chromatogramme spricht aber dafür, dass MSI eine Erhöhung des Gehaltes an freien Aminosäuren und Peptiden im Gehirn der Versuchstiere verursacht. Diese Tatsache stimmt mit der gefundenen Erhöhung des Nichteiweisstickstoffgehaltes im Gehirn nach der Injektion von MSI überein.

Aus den Ergebnissen geht hervor, dass 6 Stunden nach der Verabreichung des MSI, das heisst im praeparoxysmalen Stadium, MSI in den Stickstoffmetabolismus des Gehirns eingreift. Weil aber dem Niveau des Stickstoffmetabolismus des Gehirns, insbesondere dem Zyklus: Glutaminsäure + NH_3 ⇌ Glutamin, ein bestimmtes Niveau der funktionellen Aktivität des Gehirns entspricht (VRBA, 1955, 1956), dürfte also der Eingriff des MSI, der sich in einer Hemmung der Glutaminbildung

äussert, für eine Störung der funktionellen Aktivität des Gehirns, die in diesem Falle epileptische Anfälle zur Folge hat, verantwortlich sein.

SUMMARY

The influence of D,L-methioninesulphoximine on the nitrogen metabolism of the rat 6 hr after administration has been studied. It was found that MSI caused a decrease in the protein-N, ammonia-N and amide-N, and an increase in the nonprotein-N content of the rat brain. The total-N content was not significantly affected. In the liver, on the contrary, there was a remarkable increase in the protein-N content and a decrease in the nonprotein-N, while the content of ammonia-N and amide-N remained unchanged.

The mechanism of action of D,L-Methioninesulphoximine on the central nervous system could be explained as an inhibition of glutamine formation. The influence on the physiologically important reaction Glutamic acid + ammonia ⇌ glutamine caused a disorder of the function of the brain leading to epileptic seizures.

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THE QUANTITATIVE HISTOCHEMICAL DISTRIBUTION OF THIAMINE IN NORMAL RAT BRAIN*

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THE quantitative histochemical distribution of thiamine in normal rat brain, as reported in this communication, represents the beginning of a series of investigations on various aspects of thiamine deficiency and its effects on the nervous system. Thiamine deprivation is of particular interest for a number of reasons. First, the clinical manifestations of this state are readily produced and recognized in experimental animals, and the symptoms promptly reversed by the administration of thiamine. Second, the histopathological process selects in a reproducible fashion certain parts of the neuraxis which appear to be vulnerable to the lack of vitamin: hypothalamic nuclei adjacent to the third ventricle, and brain-stem nuclei within the floor of the fourth ventricle. Thus, thiamine deficiency in laboratory animals bears resemblance to its human counterpart. Lastly, in experimental animals deprived of thiamine, a biochemical defect has been found to follow the development of signs and symptoms of deficiency. Lowered oxygen uptake by minced brain preparations obtained from deficient animals has been reported by GAVRIPLESCU and PETERS (1931). KINNERSLEY and PETERS (1930) demonstrated increased lactic acid concentrations in the brain-stem of thiamine deficient pigeons with opisthotonus. Interest in the various roles played by thiamine in intermediary carbohydrate metabolism, as well as nerve excitation and conduction proposed by VON MURALT (1947*a*, 1957), has stimulated a number of systematic investigations presently in progress in this laboratory. An attempt is being made to evaluate the 'biochemical lesion' (a term first used by PETERS and his coworkers) associated with thiamine depletion and to attempt to correlate it with the clinic pathological changes observed. As a first step, it was deemed essential to determine the distribution of thiamine in subdivisions of the nervous system of both normal and deficient animals. Differences in content, utilization or requirements of total thiamine (free and phosphorylated) demonstrated by various areas of the brain might partially explain the inherent selectivity of the pathological process.

The quantitative determination of total thiamine in subdivisions of the nervous system has necessitated the use of a micro method easily adaptable to small samples of tissue. It is the purpose of this communication to describe this method and to report on the results obtained from thiamine estimations in nervous tissue and organs of a series of normal rats. These data are intended to serve as base line information for similar investigations on deficient animals the results of which will be reported in future publications.

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† Formerly U.S. Public Health Service Postdoctorate Research Fellow of the National Institute of Health, 1956–57.

MATERIAL AND METHODS

Wistar* female rats of 210 g average weight were used in this series of determinations. These animals were fed a normal pellet diet containing from 2·0 to 5·0 μg of thiamine per gram of diet. The animals were killed by decapitation and the organs removed and frozen immediately in a dry ice chest.

The tissue sampling technique employed in these experiments was a modification of the principle originally described by LINDERSTRØM-LANG and MOGENSEN (1938), as adapted for brain quantitative histochemistry by POPE (1952). The entire procedure was carried out in a cold room at $-12°$C. The frozen brain was cut into coronal slices measuring 2–3 mm in thickness. Cylindrical pieces of tissue from different anatomical regions were then removed from each slice by means of a metal cork borer 2 mm in diameter which was oriented at right angles to the plane of section. The cylinder thus obtained was then mounted and cut serially into twenty-one sections 40 μ in thickness with a rotary microtome. Sections no. 1, no. 11, and no. 21 were placed on an albuminized slide and used as histological control. Sections no. 5, no. 15, and no. 20 were saved for dry weight determinations. The remaining fifteen sections were used for thiamine assay, five consecutive sections being placed together in a ground glass microhomogenizer. The samples for dry weight determinations were desiccated over $CaCl_2$ and weighed on a microbalance of the Ainsworth type (sensitivity ± 1 μg). Individual brain sections weighed from 25 to 35 μg dry weight (approximately 115–160 μg fresh weight). The same sampling technique was used for determinations on heart, liver, kidney and muscle. Entire cross-sections of the cervical portion of the spinal cord were used. Small segments of sciatic nerve were removed from the lumbosacral region and sectioned into pieces measuring from 0·25 to 0·50 cm. After thorough desiccation, the pieces were weighed and duplicate thiamine determinations were carried out on aliquots of homogenate. The pieces of nerve weighed between 300 and 900 μg dry weight.

The sections saved for histological study were thawed on the slide, fixed in alcohol-formalin, rinsed in distilled water, and stained overnight with buffered thionin (WINDLE, RHINES and RANKIN, 1943).

The method used for total thiamine estimations represents an adaptation of the thiochrome method described by BURCH, BESSEY, LOVE and LOWRY (1952) for the determination of thiamine and thiamine phosphates in small quantities of blood and blood cells. In this method, the samples are homogenized and all existing enzymes inactivated by heat. Protein is precipitated with trichloroacetic acid and phosphorylated thiamine is converted to free thiamine by enzymic hydrolysis with a human prostatic acid phosphatase preparation. Oxidation of thiamine to thiochrome is accomplished with alkaline potassium ferricyanide. This is followed by the extraction of thiochrome into *n*-hexanol and measurements of fluorescence before and after exposure of the samples to an ultraviolet light source.

The sections were homogenized in 75 μl of glass redistilled† water for 1 min. From 62 to 66 μl of homogenate were transferred into a micro test tube 0·4 × 3 cm, which was tightly capped with tin foil and a small piece of rubber tubing. The tube was then heated in a water bath at 98°C for 1 min, and 140 μl of 5% trichloroacetic acid were added. The contents of each tube were then vigorously mixed with a vibrator as described by BESSEY, LOWRY, BROCK and LOPEZ (1946). After 30–40 min at room temperature, the specimens were centrifuged at 2000 rev/min for 5–10 min. Then 190 μl of the clear supernatant fluid were transferred into a test tube 10 × 75 mm (3 ml capacity), and the pH adjusted to 5·1 or 5·2 by the addition of 25 μl of 4 M-potassium acetate solution. Finally, 10 μl of acid phosphatase solution‡ were added to each specimen, and hydrolysis was carried out at 37°C for a 20 to 24 hr period. The oxidation of thiamine to thiochrome, extraction into *n*-hexanol measurements of fluorescence before and after irradiation, and the subsequent calculations of thiamine

* The animals were generously supplied by the Merck Institute of Therapeutic Research, Rahway, New Jersey.

† Glass redistilled water was used throughout the procedure. Samples of peripheral nerve were homogenized in 150 μl of water.

‡ The author is indebted to Dr. G. SCHMIDT, New England Medical Center, Boston, who generously supplied an acid phosphatase prepared from fresh frozen human prostate, as described by him (SCHMIDT, 1957). Each millilitre of undiluted enzyme preparation contained 300–400 units. The enzyme solution used for hydrolysis of cocarboxylase consists of two parts acid phosphatase to three parts glass redistilled water.

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content in the samples were carried out exactly as described by BURCH (1957). The samples were irradiated for 20 min at a distance of 6 cm from the mercury lamp.*

Throughout the procedure, the samples were covered with parafilm to protect them from dust. Great care had to be exercised to prevent the liquid contents of the samples from coming into contact with the parafilm cover, since erroneously high fluorometer readings would have resulted. The final calculations of thiamine content of individual samples were made by assuming that the dry weight values obtained from the sections selected for weight control were representative for all of the sections in the same tissue cylinder. The results were expressed as micrograms of thiamine per gram dry weight of tissue. Each figure represents the average of triplicate determinations carried out on each area sampled.

RESULTS

BURCH *et al.* (1952) have presented both biological and physicochemical evidence validating their method for blood. Some of these experiments were repeated substituting tissue for blood, and similar results were obtained. The reproducibility of the method was tested by determining the total thiamine content of twelve consecutive samples of liver cut from the same block of tissue. The values were found to average 22·9 μg $\pm$ S.D. 1·6 per gram dry weight. This represents a reproducibility of $\pm$ 7 per cent. Recovery experiments were done to ascertain the accuracy of the method. In these experiments, the thiamine hydrochloride and cocarboxylase were added to various specimens; the results are shown in Table 1. Full recovery of thiamine after the addition of cocarboxylase would suggest that hydrolysis by the acid phosphatase preparation used in the experiments is virtually complete after 20 to 24 hr. This was previously noted by LOHMANN and SCHUSTER (1937) who used a similar enzyme preparation.

TABLE 1.—RECOVERY OF THIAMINE AND THIAMINE PYROPHOSPHATE
(Thiamine hydrochloride or thiamine pyrophosphate in aqueous solution was added to the specimen prior to hydrolysis with acid phosphatase.)

Sample	Total thiamine (initial) (mμg)	Substance added	Amount added† (mμg)	Total thiamine found (mμg)	Recovery (%)
Spinal cord	0·6	Thiamine hydrochloride	2·5	3·2	103
Cerebral cortex	0·9	Thiamine hydrochloride	2·5	3·2	94
Liver	4·4	Thiamine hydrochloride	2·5	6·7	97
Spinal cord	1·2	Thiamine pyrophosphate	2·5	2·9	99
Cerebral cortex	1·6	Thiamine pyrophosphate	2·5	3·5	102
Liver	1·2	Thiamine pyrophosphate	2·5	3·0	102

† 2·5 mμg of thiamine pyrophosphate are equivalent to 1·76 mμg of thiamine hydrochloride.

The results obtained from total thiamine determinations of normal rat brain, liver, heart, kidney and muscle are summarized in Table 2. The data represent the average of estimations carried out on seven different animals and are expressed as micrograms of thiamine per gram dry weight of tissue. The thiamine content of heart was the highest, followed by kidney, liver, and brain; muscle contained the least. Comparison of the foregoing results with similar observations published by

* General Electric mercury lamp H 250–A5 and transformer 9T64Y–1017 were used.

others, using either a fermentation or thiochrome method, is shown in Table 3. In order to compare data, average dry weight figures had to be converted to grams fresh weight by using known values for percent solid matter content of brain,

TABLE 2.—DISTRIBUTION OF TOTAL THIAMINE IN NORMAL RAT BRAIN, LIVER, KIDNEY, HEART AND GASTROCNEMIUS
(The results are expressed as micrograms of thiamine per gram dry weight.)

Organ	Seven animals		
	Range (μg/g)	Average (μg/g)	S.D.
Brain*	10·8–16·4	13·7	±1·2
Liver	21·6–41·5	30·0	±6·1
Heart	25·5–49·9	36·8	±7·9
Kidney	21·8–39·5	30·3	±5·5
Gastrocnemius	7·1–12·5	9·6	±1·6

* Brain data represent an average figure of ten areas of the brain as shown in Table 4.

TABLE 3.—TOTAL THIAMINE VALUES IN RAT TISSUES AS REPORTED BY OTHER INVESTIGATORS
(The values are expressed as μg/g fresh weight.)

Reference	Method	Brain	Liver	Kidney	Heart	Muscle	Sciatic nerve
MITCHELL and ISBELL (1942)	Fermentation	3·0	7·6	4·2	7·3	1·8	
OCHOA and PETERS (1938)	Fermentation	2·9–3·1	5·5–6·0		3·5–4·2	0·9–1·3	
SCHULTZ *et al.* (1939)	Fermentation	2·5–4·2	10·0	6·7	7·2	1·3	
RITSERT (1939)	Thiochrome	2·5–3·5	4·0–8·0	3·5–6·0	1·0–3·0	0·8	
DE CARO and BUTTURINI (1940)	Thiochrome	3·5–4·5	4·5–14			0·9	
VON MURALT (1947b)	Thiochrome	3·1					1·6
WIEDENBAUER (1938)	Thiochrome	2·2–3.2	2·8–7·1	7·6	2·2–3·5		
WESTENBRINK and GOUDSMIT (1938)	Thiochrome	6·0	7·0–13	11		1·5–5·0	
Author*	Thiochrome	3·0	9·0	7·6	7·4	2·3	1·5

* Dry weight values were converted to fresh weight by assuming the percent solid matter to be as follows: brain 22%, liver 30%, heart 20%, kidney 25%, muscle 24% and peripheral nerve 36%.

peripheral nerves and other organs (see footnote, Table 3). Some degree of variation is noted in the results obtained in other laboratories. This is partially due to the fact that different types of methods were employed. The thiamine content of brain and other organs, as determined by the above method, is in good agreement with the data published by others.

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Total thiamine determinations of selected areas of normal rat brain, spinal cord and sciatic nerve of 8 different animals are summarized in Table 4. From these results, it is evident that the distribution of total thiamine in the brain of normal rats is fairly uniform. A relatively wide range is noted for each subdivision. The brains with either a high or low thiamine content for one area did not necessarily show corresponding differences in other parts. Statistical analysis of the results reveals that the cerebellar vermis is significantly higher in its thiamine content than the remainder of the brain, whereas the thalamus is significantly lower ($P < 0{\cdot}01$ in each case, according to FISHER's (1946) *t*-test for small samples).

TABLE 4.—DISTRIBUTION OF TOTAL THIAMINE IN NORMAL RAT BRAIN, SPINAL CORD AND SCIATIC NERVE

(The results are expressed as micrograms of thiamine per gram dry weight.)

Area sampled	Thiamine content		
	Range (μg/g)	Average (μg/g)	S.D.
Cortex	9·0–16·7	13·0	±2·5
Caudate nucleus	10·9–25·7	17·4	±4·5
Thalamus	5·8–12·3	9·2	±2·3
Hypothalamus	8·1–17·8	13·0	±3·2
Mammillary region	8·6–16·0	12·4	±2·4
Periaqueductal region	7·0–19·0	13·2	±3·5
Lateral pontine tegmentum	4·7–17·1	11·5	±4·7
Base of pons	7·3–16·4	13·1	±2·5
Vermis	14·6–25·7	21·1	±4·2
Medulla	7·6–17·7	13·4	±3·1
Spinal cord*	4·6–11·9	8·4	±2·4
Sciatic nerve†	2·9–5·6	4·1	±0·9

* Six animals.
† Nine animals.

The large range noted for each area probably reflects the biological variation from animal to animal and the lack of exact reproducibility in the sampling procedure. The histological control sections revealed that in each experiment the same anatomical site had been sampled, but that the proportion of neurones and glial elements to fibre tracts differed considerably. Because of technical difficulties, no attempt was made to correlate quantitatively the structural composition of the sample with the thiamine content of the region under study.

DISCUSSION

The results obtained from the foregoing series of experiments provide additional information regarding the content of total thiamine in various subdivisions of the intact nervous system. Similar data are partially contained in the publications of VILLELA, DIAS and QUEIROGA (1949), FEREBEE, WEISSMAN, PARKER and OWEN (1943), and IIZUKA (1954). The results of such experiments unfortunately do not clarify the biochemical problems associated with thiamine deficiency. There appears to be little, if any, correlation between total thiamine content of any given subdivision of

the brain and its susceptibility to thiamine deprivation. The heart, brain and peripheral nerves are known to be exceedingly vulnerable to thiamine deficiency, yet only the heart contains a high concentration of total thiamine, the brain and peripheral nerves, respectively, revealing intermediate and low concentrations. In the depleted animal, the lateral pontine tegmentum is the subdivision of the brain showing the most severe histopathological alterations.* In the normal animal, however, this region appears to be average in its total thiamine concentration (Table 4). Thus, one must assume that tissue levels probably do not reflect the selective vulnerability of certain parts to thiamine deficiency. Each organ or part of the nervous system appears to have its particular thiamine levels for depletion. The first of these is characterized by minimal symptoms and reversible structural and metabolic changes. The second, considerably lower, marks the advent of striking symptoms attributable to loss of function accompanied by irreversible pathological changes.

The significantly higher total thiamine content noted in the cerebellar cortex appears to be confirmed by IIZUKA (1954) who demonstrated a high concentration of thiamine in the Purkinje cells of the cerebellar cortex by means of a fluorescence microspectrographic technique developed by SJÖSTRAND (1946). According to FEREBEE *et al.* (1943), the level of thiamine in human brain is also highest in the cerebellum. These observations would appear to indicate that the cerebellum possesses a relatively high metabolic rate. DIXON and MEYER (1936) have shown a considerably higher O_2 uptake by bovine cerebellar cortex compared to other parts of the brain. HIMWICH and FAZEKAS (1941) confirmed this finding in canine cerebellum, but failed to observe it in rat brain (HIMWICH, 1951). This may be due to the fact that the entire rat cerebellum was used in these experiments. Whole cerebellum preparations would tend to contain more white matter than cerebellar cortex: thus, one might expect the former to give lower oxygen uptake figures. Further evidence of higher metabolic activity in the cerebellum was suggested by HESS and POPE (1953) who found higher cytochrome oxidase activity in rat cerebellum, as compared to other parts of the nervous system. A high thiamine content could easily be accounted for by the cellular composition of the cerebellum with its great nerve cell density and consequent large actively metabolizing mass per unit volume of tissue. Tissue thiamine levels, however, do not appear to parallel the activity of enzymes concerned with glycolysis and respiration. ROBINS *et al.* (1956, 1957) noted that the activity of fumarase lactic dehydrogenase, and malic dehydrogenase was generally higher in a monkey cerebral cortex when compared to cerebellar cortex. Aldolase activity, however, seemed higher in the cerebellar cortex. Lastly, GAVRIPLESCU and PETERS (1931) noted that the cerebellum of athiaminotic pigeons did not show the reduction of O_2 uptake found in other parts of the brain. This could be ascribed to higher levels of thiamine in the cerebellum.

At the present time, no reasonable explanation can be offered for the significantly lower levels of thiamine found in the thalamus, since the literature contains little biochemical information regarding this subdivision of the brain.

Measurements of total thiamine in tissues of animals at various stages of deficiency are presently in progress and will be the subject of a subsequent communication. It is hoped that information relating to possible patterns of depletion and replenishment

* Histopathological studies on thiamine depleted animals are presently in progress and will be the subject of a separate communication.

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by various subdivisions of the nervous system, may in part, elucidate their selective vulnerability to the lack of thiamine. These experiments supply data concerning thiamine as coenzyme of the carboxylase and transketolase systems; they, however, fail to contribute information regarding the apoenzyme part of these enzyme systems. A constant ratio of coenzyme to apoenzyme may well be of the utmost importance in the maintenance of the integrity of the nervous system. Selective vulnerability may be partially explained by an imbalance of this ratio in the parts of the brain most readily afflicted by thiamine deficiency.

SUMMARY

The total thiamine (free and phosphorylated) content of heart, liver, kidney, muscle, sciatic nerve and ten subdivisions of the brain of normal Wistar rats has been measured by a quantitative fluorometric method. Results have shown the method to be specific, reproducible and accurate.

Thiamine is evenly distributed throughout the brain with the exception of the cerebellum which has a higher concentration, and the thalamus which contains less thiamine than the remainder of the brain.

It is suggested that the thiamine content of the cerebellum is related to its histological structure and its metabolic rate.

There appears to be no correlation between tissue thiamine levels and susceptibility by certain parts of the neuraxis to vitamin B_1 deprivation.

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STUDY OF ESTERASE ACTIVITY IN HUMAN BRAIN AND SERUM*

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IN order to resolve the complex esterase activity of tissues, techniques involving differential inhibition by various agents have been employed as well as analysis of kinetic data. ALDRIDGE (1953) has distinguished with the aid of organophosphorus inhibitors between an A-esterase and a B-esterase which seem to resemble the aromatic and aliphatic esterases of MOUNTER and WHITTAKER (1953). In addition, MYERS (1956) has reported a DINP-resistant and a DINP-sensitive esterase, as well as a tributyrinase which is DINP-resistant. BERGMAN, SEGAL and RIMON (1957) have also found a C-esterase resistant to DFP. HOFSTEE (1954), on the other hand, has differentiated esterases by analysis of kinetic data. In the absence of purified enzyme preparations, such techniques can offer valuable information to provide a basis for understanding the nature and function of these enzymes. However, the degree of selective inhibition by these specific inhibitors is always problematic, and the amount of overlapping is difficult to evaluate.

ALDRIDGE (1954) has already pointed out that brain esterases are different from the esterases of serum. In this report an analysis of the esterase activity in human brain is presented in which differential activity is established by means of the stability of the esterase activity, and the distinctions between brain and serum esterases are based on physical properties and substrate characteristics. In addition, the esterase activity of human brain, localized in particulate brain areas, was surveyed. The role of esterases in Wallerian degeneration (LUMSDEN, 1952), and as a potential aetiological factor in demyelination (BRICHNER, 1930), makes such a study desirable.

EXPERIMENTAL

Cholinesterase activity was inhibited by performing all assays in the presence of 5×10^{-5}M-eserine sulphate, so that only eserine-resistant enzymes were studied.

Methods

Esterase activity was measured manometrically by a modification of the method of AMMON (1933), using PhAc and PhBu as substrates. These compounds were found to be highly active substrates for nervous tissue esterases.

Abbreviations:
DINP = di*iso*propyl-*p*-nitrophenylphosphate
DFP = di*iso*propylfluorophosphonate
PhAc = phenyl acetate
PhBu = phenyl butyrate

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The tissues were homogenized in 0·023 M-$NaHCO_3$–0·154 M-NaCl. A 1 : 10 homogenate (0·1 ml) was employed for assaying grey matter and, for white matter the same concentration of a 1 : 5 homogenate was used. Serum was usually diluted 1 : 100 for assay. The enzyme was pipetted into the main compartment and pre-incubated with eserine sulphate for 20 min in a 95% N_2–5% CO_2 atmosphere. The final concentration of eserine sulphate in the kinetic and stability studies was 1×10^{-6} M, but the distribution data were obtained using 5×10^{-5} M, to provide a satisfactory margin of safety. Substrate (1·0 ml) was then tipped in from the side-arm and CO_2 evolution measured at 5 or 10 min intervals after 5 min equilibration. The hydrolysis generally followed a zero-order reaction for the 30–60 min experimental period. Assays were conducted at 37°C and corrected for non-enzymic hydrolysis. The total volume of the reaction mixture was 2 ml.

PhAc and PhBu were synthesized by condensing phenol with the acid chloride and washing with 5% Na_2CO_3, and the ester then distilled The purity of the substrate was determined manometrically using excess serum as an enzyme source

All the data reported were obtained on human material The brain tissue was obtained from adult males who had expired from non-neurological complications On post-mortem examination it was confirmed by one of us (E. L.) by visual observation of the sections to be assayed and by histological preparation of the brain tissue that no pathological tissue was present The specimen was removed 6–8 hr after death and stored at −15°C until ready for use, at which time it was allowed to remain at room temperature until the cortex could be dissected and the remainder of the brain cut. The sections were wrapped in pliofilm and returned to the deep-freeze until required.

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RESULTS

Stability in situ. The stability of the esterase activity *in situ* at room temperature and stored at −15°C in the deep freeze is shown in Table 1. After standing overnight

TABLE 1.—STABILITY OF BRAIN ESTERASE ACTIVITY *in situ*

Time (days)	Temp. (°C)	PhAc (μmoles/g per hr)	Δ (%)	PhBu (μmoles/g per hr)	Δ (%)	PhAc / PhBu
White matter						
0*	—	397	—	344	—	1·15
14	−15	366	−7	324	−6	1·13
28	−15	366	−7	326	−6	1·12
Grey matter						
0	—	857	—	509	—	1·68
20 hr	+26	772	−10	513	+1	1·50

* Zero time is the time of receipt of the specimen.

at room temperature, a decrease of 10 per cent in PhAc activity was observed, while practically no change in PhBu activity occurred. Under freezing conditions the loss in activity was 7 per cent and 6 per cent, respectively, after 28 days of storage. The similarity in the degree of activity lost for the two substrates is reflected in the constancy of the PhAc/PhBu ratios. These results indicate the relative resistance of esterases to destruction under these conditions, and provide a basis for the type of distribution study reported here later.

Effect of eserine. The inhibitory effect of eserine sulphate on PhAc and PhBu hydrolysis in serum and blood is shown in Fig. 1(a, b). The data shows that at 5×10^{-5} M-eserine, about 10 per cent of the PhAc hydrolysis is inhibited in brain

and less than this amount for PhBu hydrolysis. Distinctions that appear in the I_{50}* values for the tissues and for the two substrates may be the result of different amounts of tissue employed for the assay. Therefore, the inert-protein-binding effect may vary rather than reflect differences in eserine-inhibitory properties.

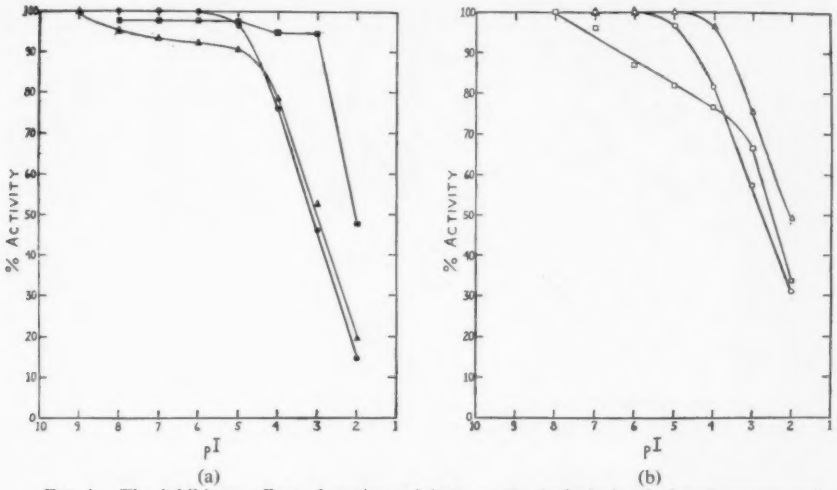

FIG. 1.—The inhibitory effect of eserine sulphate on the hydrolysis of phenyl acetate and phenyl butyrate. Values are plotted as a per cent of the non-inhibited activity vs. the negative logarithm of the inhibitor concentration.
(a) Phenyl acetate: ■ serum, ● cerebral cortex, ▲ white matter.
(b) Phenyl butyrate: □ serum, ○ cerebral cortex, △ white matter

Effect of Ca^{2+}*, gum acacia and dialysis on esterase activity in brain.* The activating effect of Ca^{2+} on esterase activity has been reported by SCHONHEYDER and VOLQVARTZ (1945) and by BERNSOHN, ZELLER and COCHRANE (1954), and gum acacia has been used to protect the enzyme and emulsify the substrate as reported by ARCHIBALD (1946). In determining the effect of these substances, as well as the effect of dialysis on grey and white matter of brain, a comparison with human serum was made. These results are shown in Table 2. The hydrolysis of PhAc by serum is activated by 5×10^{-4} M-Ca^{2+}, increasing from 3562 to 7725 μmoles/ml per hr, an increase of 117 per cent. Gum acacia at a 1 per cent level resulted in a further increase in activity to 9460 μmoles/ml per hr, a 166 per cent increase, whereas the simultaneous addition of Ca^{2+} and gum acacia resulted in no significant increase above that obtained by gum acacia alone. In contrast to these results the hydrolysis of phenyl butyrate by serum was not significantly activated by either Ca^{2+} or gum acacia, or by a combination of both. This would indicate also that substrate solubility was not a limiting factor in these experiments.

On dialysis, at 2°C, differences in the behaviour of PhAc and PhBu also were apparent. No loss in esterase activity toward both substrates was found on 40–48 hr dialysis against distilled water, but the PhAc activity again could be stimulated by the addition of Ca^{2+}, whereas no such reactivation appeared with PhBu as a substrate. The control samples were allowed to stand under the same conditions as the dialysed preparations before being assayed. In contrast, the esterase activity of human grey

* Concentration of inhibitor required to produce a 50 per cent inhibition in activity.

TABLE 2.—EFFECT OF DIALYSIS, Ca^{2+} AND GUM ACACIA ON ESTERASE ACTIVITY IN HUMAN SERUM AND BRAIN

	PhAc	PhBu	$\frac{PhAc}{PhBu}$
Serum	3562	260	13·7
Serum + 5×10^{-4} M-Ca^{2+}	7725	273	28·2
Serum + 1% acacia	9460	290	32·6
Serum + Ca^{2+} + 1% acacia	9730	290	33·6
Serum (dialysed)	3394	224	15·2
Serum dialysed + Ca^{2+}	7492	276	27·1
Cerebral cortex	1239	433	2·86
Cerebral cortex + Ca^{2+}	1252	442	2·83
Cerebral cortex (dialysed)	1134	429	2·64
Cerebral cortex dialysed + Ca^{2+}	1154	444	2·60
White matter	295	134	2·20
White matter + Ca^{2+}	295	156	1·89
White matter + 1% gum acacia	290	196	1·56
White matter (dialysed)	261	153	1·71
White matter + Ca^{2+}	258	157	1·64

Activity is expressed as μmoles/g or ml/hr.
Dialysis was carried out against distilled water at 2°C, and the undialysed sample was prepared simultaneously and kept under similar conditions for the duration of the dialysis experiment before being assayed.

and white matter of brain showed no activating effect of either Ca^{2+} or gum acacia with either PhAc or PhBu as a substrate. Dialysis also did not appreciably change the esterase activity with either PhAc or PhBu as substrates, and there was no significant loss of activity on dialysis, or augmentation of PhAc or PhBu hydrolysis by Ca^{2+} after dialysis.

The differences between serum and brain are further emphasized when the PhAc/PhBu ratios are compared. In grey matter this ratio varied from 2·6 to 2·86, under these experimental conditions. In white matter this range was 1·64–2·20. However, in serum this ratio was 13·7, which increased to 28·2 when activated with Ca^{2+} and further increased to 32·6 with the addition of gum acacia. This demonstrated the effect of these substances on PhAc activity and the relative insensitivity of the PhBu-hydrolysing enzyme in serum.

Decay of esterase activity in homogenates. Homogenates, prepared and stored in the refrigerator at 2°C, and whose activity was followed periodically over a 21 day period, showed varying lability toward PhAc and PhBu hydrolysis in brain. In homogenates of cerebral cortex, the PhAc activity declined only slightly for the first 3 days in storage, amounting to only 83 μmoles/g per hr, or 7 per cent of the original activity. The PhBu activity diminished more rapidly decreasing 13, 35 and 30 per cent of the original activity for each successive day of the same time interval. Over the following days, the rate of decline of enzymic activity for both substrates was uniform, averaging about 1–1·5 per cent per day, so that subsequent to the initial 3 day period the loss of esterase activity toward both substrates was similar, averaging 18 per cent of the activity of the third day (Table 3).

Because of the relative lability of the PhBu hydrolysing enzyme during the first three days, the ratio of PhAc/PhBu which initially was 1·63, increased rapidly to

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TABLE 3.—DECAY OF ESTERASE ACTIVITY* IN BRAIN HOMOGENATES OF HUMAN CEREBRAL CORTEX STORED AT 2°C

Time (days)	PhAc	Δ (%)	PhBu	Δ (%)	PhAc / PhBu
0	1140	—	701	—	1·63
1	1143	0	612	−13	1·87
2	1112	−2	523	−25	2·13
3	1057	−7	492	−30	2·15
7	1028	−10	432	−38	2·38
9	1020	−11	412	−41	2·47
14	993	−13	396	−44	2·51
21	866	−24	364	−48	2·38

* μmoles of substrate hydrolysed per g of wet tissue per hr.

2·15 on the third day and remained relatively constant thereafter, averaging 2·38 on the twenty-first day of the experiment.

The esterase decay pattern of white matter differed from that of cerebral cortex (Table 4). The same initial loss in PhBu hydrolysing activity was observed, amounting

TABLE 4.—DECAY OF ESTERASE ACTIVITY* IN BRAIN HOMOGENATES OF WHITE MATTER STORED AT 2°C

Time (days)	PhAc	Δ (%)	PhBu	Δ (%)	PhAc / PhBu
0	366	—	325	—	1·13
1	326	−11	232	−29	1·40
2	313	−14	219	−33	1·43
5	295	−19	174	−46	1·70
9	254	−31	134	−59	1·90
14	234	−36	98	−70	2·39
21	234	−36	94	−71	2·48

* μmoles of substrate hydrolysed per g of wet tissue per hr.

to 33 per cent of the initial activity in a 2 day period. However, this high rate of decay did not diminish as in cerebral cortex, and after 21 days, 71 per cent of the initial activity had been lost, compared to the value of 48 per cent obtained in grey matter. The PhAc hydrolysis, although not as marked as that of PhBu, also showed a greater rate of decay than that observed in cerebral cortex.

Another distinction between the two tissues was noted, in that the initial PhAc/PhBu ratio in white matter was 1·13, and this gradually increased to 2·48 on the final experimental day, the latter value comparable to the maximum figure obtained in grey matter.

Kinetics of esterase hydrolysis. The concentration–activity curves for the esterases of serum and brain with PhAc and PhBu as substrates are shown in Fig. 2. The increasing rate of PhAc hydrolysis with concentration is such that at approximately

5×10^{-3} M the PhAc/PhBu ratio is unity for grey matter, whereas at 2×10^{-2} M this ratio is 1·6. In addition, despite the similar stability characteristic of aged homogenates of cerebral cortex toward PhAc and PhBu hydrolysis, the pS curves differ. Since the substrates are from an homologous series, such differences may indicate hydrolysis by different enzymes. PhAc hydrolysis increases linearly with increasing substrate concentration, whereas PhBu hydrolysis by aged homogenate seems to

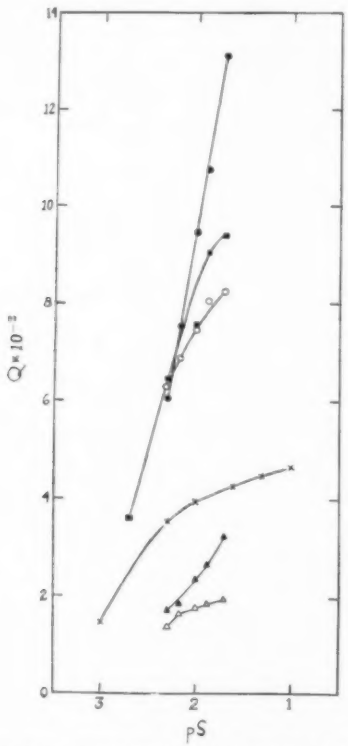

FIG. 2.—Esterase activity of serum and brain. Q = μmoles of substrate hydrolysed per g of wet tissue per hour, pS = negative logarithm of the substrate concentration. Phenyl acetate: ■ serum, ● cerebral cortex, ▲ white matter; phenyl butyrate: ○ cerebral cortex, △ white matter; ×, aged cerebral cortex homogenate.

simulate a first-order reaction. That this result was not due to solubility factors was determined by emulsifying the substrates with Triton X-100.* This surface-active agent reduced the enzymic activity by about 16 per cent, over the substrate–concentration range, but did not alter the shape of the curves shown, for either substrate. The change in PhAc/PhBu ratio for white matter with changing substrate concentration is apparent. Treatment of the data according to LINEWEAVER and BURK (1934), (Fig. 3) shows that the esterase activities for both substrates and the tissues studied, follow a linear relationship with the exception of the PhBu-activity for human serum. K_m and V_m values for the esterase are shown in Table 5. These data are presented only to demonstrate that the substrate hydrolysis rates do follow these

* Kindly supplied by Rohm & Haas Co.

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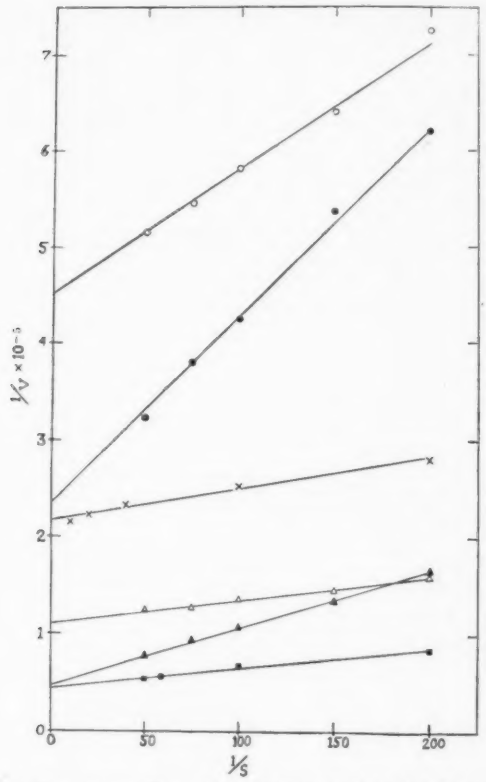

FIG. 3.—LINEWEAVER–BURK plot of esterase activity in brain and serum.
V = moles per g per hr, S = substrate concentration
(symbols same as in Fig. 1).

relationships and imply only that the hydrolysis is a result of enzymic activity which follows these laws. The observation of HOFSTEE (1952) regarding the representation of the data, as well as the range of substrate concentration studied, would preclude any conclusions concerning the unitary nature of the esterase activity.

TABLE 5.—K_m AND V_m CONSTANTS FOR ESTERASE ACTIVITY IN SERUM AND BRAIN

	K_m	V_m
Phenyl acetate		
Cerebral cortex	$1{\cdot}2 \times 10^{-3}$	$2{\cdot}1 \times 10^{-3}$
White matter	$8{\cdot}0 \times 10^{-3}$	$4{\cdot}2 \times 10^{-4}$
Serum	$3{\cdot}7 \times 10^{-3}$	$1{\cdot}1 \times 10^{-2}$
Phenyl butyrate		
Cerebral cortex	$2{\cdot}1 \times 10^{-3}$	$9{\cdot}0 \times 10^{-4}$
Cerebral cortex (aged)	$1{\cdot}8 \times 10^{-3}$	$4{\cdot}6 \times 10^{-4}$
White matter	$2{\cdot}9 \times 10^{-3}$	$2{\cdot}2 \times 10^{-4}$

Esterase activity in brain. The localization of PhAc hydrolysis in various functional areas of human brain is shown in Table 6. Analysis of this data indicates that there exists a true localization of this activity in the neuronal elements. All grey matter areas with the exception of the hippocampus show a uniformly high activity ranging from 698 μmoles/g per hr in the occipital grey, to 851 μmoles/g per hr in the orbital

TABLE 6.—DISTRIBUTION OF ESTERASE ACTIVITY IN AREAS OF THE HUMAN BRAIN

	No. of specimens assayed	Activity ± S.D. (μmoles/g per hr)
Cerebral cortex		
Orbital pole	10	851 ± 204
Temporal	8	829 ± 112
Pre-central	10	803 ± 163
Post-central	8	755 ± 157
Occipital	9	698 ± 208
Frontal	10	786 ± 148
Parietal	5	796 ± 108
Insula	5	768 ± 128
Operculum	4	844 ± 78
Cingulate	10	730 ± 112
Hippocampus	8	478 ± 60
Diencephalon		
Thalamus	9	656 ± 110
Hypothalamus	8	518 ± 133
Cerebellum		
Cerebellar cortex	10	710 ± 146
Dentate nucleus	6	455 ± 126
Basal ganglia		
Putamen	9	971 ± 152
Caudate nucleus	10	920 ± 179
Pallidum	8	719 ± 103
Brain stem		
Pons	8	470 ± 79
Substantia nigra	9	594 ± 96
Red nucleus	5	442 ± 80
White matter		
Occipital	8	352 ± 98
Frontal	9	280 ± 98
Corpus callosum	10	283 ± 31
Cerebellum	8	305 ± 68
Internal capsule	10	409 ± 133
Medulla		
Floor, 4th ventricle	8	413 ± 112
Pyramidal tract	8	319 ± 60

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pole. The basal ganglia areas ranged from 719 μmoles/g per hr in the pallidum to 971 μmoles/g hr in the putamen, the latter being the highest value obtained. The values for the cerebral cortical areas are noteworthy for their uniformity. The white matter areas are uniformly low, with the internal capsule value of 409 μmoles/g per hr having the highest activity. It is probable that this area may be contaminated with some grey substance. Other white areas yielded lower values which were about 30 per cent of the grey matter esterase activity.

DISCUSSION

The complexity of the esterase activity which has been shown by the use of specific inhibitors is further demonstrated in this study on their chemical and physical properties. The inhibitors used by other workers were unavailable in this investigation and consequently correlations between properties and behaviour toward inhibitors were not studied. The data obtained here on brain and serum would tend to indicate that a resolution of the problem probably requires the utilization of purified enzyme preparations.

On the basis of these results, it appears that serum, as distinct from brain, has an esterase which hydrolyses PhAc and which is Ca^{2+} dependent, and is protected by gum acacia. Since the addition of both Ca^{2+} and gum acacia to the media did not enhance the enzymatic activity beyond that obtained with gum acacia alone, it is presumed that the activating effect of both substances are for the same enzyme. This esterase is distinct from the PhBu hydrolysing enzyme which is Ca^{2+}, or gum acacia-independent. The activity of the serum esterase after dialysis indicates a PhAc/PhBu ratio of 13·8, whereas this ratio in cerebral cortex is 2·4, and 1·13 in white matter. This variability in ratio can only be attributed to the fact that more than one enzyme is responsible for the hydrolysis of these two substrates, and that their distribution pattern differs for the three tissues. After loss of the PhBu-labile enzyme, the PhAc/PhBu ratios for grey and white matter are similar, indicating either that the hydrolysis of these two substrates in both types of tissue is accomplished by the same enzyme, or that the distribution pattern of enzyme responsible for the hydrolysis of these two substrates is similar. While analysis of the data indicates that the enzyme–substrate reaction follows a Michaelis–Menten relationship, HOFSTEE (1952), has discussed the limitations of this type of analysis under the experimental conditions reported here, and while the data support the idea that each substrate is hydrolysed by one enzyme, this conclusion cannot be drawn without additional study. Some experiments with mixed substrates gave results which indicated that at low concentrations hydrolysis was additive, indicating that two esterases were involved.* This is in contrast to the results of UNDERHAY (1957) on phenyl and indoxyl esters in red cells and plasma, where additive experiments indicated that only one enzyme was involved in the hydrolysis of these substrates.

It seems, therefore, that at least four esterases are shown to be responsible for the hydrolysis of PhAc and PhBu. In serum there is a Ca^{2+} dependent, PhAc hydrolysing enzyme, and an enzyme responsible for PhAc and PhBu hydrolysis. Whether the latter is the same or two different enzymes is not apparent, except that PhAc hydrolysis follows a Michaelis–Menton relationship, whereas, PhBu hydrolysis does not. Then in brain a PhBu-labile hydrolysing enzyme is present. The same

* Unpublished observations.

remarks in regard to the remaining PhAc and PhBu hydrolysis that were made in respect to serum apply to brain, except that the situations in serum and brain differ by virtue of the different PhAc/PhBu ratios in these two tissues. Therefore, either brain or serum or both have two different enzymes which hydrolyse PhAc and PhBu at different rates.

The study on the distribution of PhAc activity reveals that the esterase activity is correlated with the grey matter. This is in agreement with the findings of BARRNETT and SELIGMAN (1953). The uniformity of activity in all cerebral grey matter area would lead to a tentative conclusion that this activity is not correlated with cell number as is the case with many other enzyme systems. This would tend to show that the enzymic activity is not related to nuclear function. ZACKS and WISLOCKI (1953) in their study on placental esterases have surmised that these enzymes may play a role in the transfer of substances across the placental barrier, and it may be that these findings indicate a function of the enzyme in membrane metabolism. This association of activity with the grey matter areas, and the relative low activity in white matter would imply that the role of the esterases in demyelination may not be important, since one would expect alterations in the grey matter to occur at least concurrently with changes in the white matter, if changes in esterase activity were an etiological factor in demyelinating diseases.

While PhAc and PhBu have been employed in this study, because the rate of reaction enables one to make observations which are difficult with other esters which are relatively inactive, it is of course of paramount importance to attempt to ascertain the physiological substrates for this class of enzymes. While it is easy to speculate on this problem, there is nothing in this study to indicate what these substrates may be.

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SUMMARY

A study of the chemical and physical properties of esterases in human brain and serum shows that at least four different eserine-resistant esterases occur in these tissues. These are (a) a Ca^{2+} dependent enzyme in serum which hydrolyses phenyl acetate, (b) a phenyl butyrate-hydrolysing enzyme of brain which is labile on ageing, (c) and (d), at least two different enzymes in serum and brain which hydrolyse phenyl acetate and phenyl butyrate, as can be demonstrated by the different relative activity for the two substrates employed.

A distribution study of esterase activity in brain areas demonstrated that high phenyl acetate hydrolysis is associated with the grey matter, both cortical and sub-cortical.

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ELECTROPHORETIC PROTEIN FRACTIONS AND THE HYDROPHILIC PROPERTY OF BRAIN TISSUE—I

THE PROTEINS OF NORMAL BRAIN

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INTRODUCTION

IN a previous study of the protein fractions of the dog brain, it was shown that in the immature brain the soluble proteins form the major portion of the total solid matter and that their content gradually decreases with age (KIYOTA, 1956). The insoluble protein fraction showed a consistent increase during myelination. Fractions of the soluble proteins obtained by electrophoresis showed changes with growth soon after birth. The changes with age of the soluble protein fractions of different solubility are closely linked with the variation with age of the hydrophilic property of brain tissue. However, the relation between these protein fractions and the hydrophilic property of different regions of human brain, and the chemical nature of the proteins, remained to be elucidated. It is the purpose of the present paper to clarify some of these issues as studied in human material. A preliminary report of these studies has appeared elsewhere (KIYOTA, 1957).

MATERIALS AND METHODS

Brains of ten healthy adults who died of traumatic wounds were selected. The brain samples were taken within 6 hr after death and revealed no pathological changes. The cerebral hemisphere was removed from the pia mater avoiding contamination with blood as much as possible. Material was taken mainly from the frontal and parietal lobes, as no significant differences were found in the protein distribution amongst the various lobes of the cerebrum.

The total water content of the tissue was measured by drying at 100°C for 24 hr. The total nitrogen of the dry matter was determined by the Kjeldahl technique. A constant wet weight of tissue was homogenized and diluted six times with 0·05 M-phosphate buffer, pH 8. A part of the supernatant obtained after centrifugation of the homogenate at 14,500 **g** (temperature kept below 17°) was used for the measurement of the soluble protein and of the non-protein nitrogen extracted with trichloroacetic acid (final concentration, 0·12 N). The amount of protein was calculated by multiplying protein nitrogen values by 6·25. All values are expressed as per cent of the wet weight of tissue. Samples of the supernatant were also dialysed against phosphate buffer for 48 hr in the cold and the precipitated protein was removed by centrifuging at 14,500 **g**. The supernatant was then fractionated in the Tiselius electrophoresis apparatus using the *Schlieren* scanning method. The electrophoretic conditions were as follows: 0·05 M-phosphate buffer, pH 8·0; 140–150 V, 11 MA; time of run, 60 min.

For the paper electrophoresis, the brain extracts were made 1 : 1 or 1 : 2 (w/v) with phosphate buffer and proteins were separated on the Grassmann apparatus (GRASSMANN and HANNIG, 1952). The electrophoretic conditions were as follows: 0·05 M-phosphate buffer, pH 8·0; 10–12 V/cm,

0·5–1·0 mA/cm; time of run, 15 hr; paper, no. 2 filter paper (obtained from Toyo-Roshi Co.); dye solution: 0·05% bromphenol blue containing 1·0% mercuric chloride and 2% glacial acetic acid. After dyeing, the paper was immersed into heated wax and the absorbancy of the staining on the paper was measured directly in an electrophotometer.

RESULTS

1. *Water and protein content*

The protein accounted for about half of the dry weight in the cortex and about one-third of the dry weight in the white matter. When the 'soluble protein' was determined, the content of the 'insoluble protein' not extracted under the same conditions could be calculated. Thus, the tissue proteins were divided in two groups of different solubility.

The ratio of soluble to insoluble protein was about 1 : 2 in the cortex, while it was about 1 : 4 in the white matter. The ratio of soluble protein to dry matter was 17·5 per cent in the cortex and 7·2 per cent in the white matter. This shows that the insoluble proteins and lipids are predominant in the white matter, while the soluble protein is predominant in the cortex. Part of the proteins and lipids in the brain exist in a dynamic state and are rapidly renewed (SCHOENHEIMER, 1946), while another part appears to migrate to and from the periphery of the cerebrum. The rest of the lipids and proteins seem to be relatively inert and are of structural importance. Insoluble proteins, with lipids, seem to be the main constituents of myelin, as they were found to increase concomitantly with myelination during the maturation of the cerebral hemispheres in the dog and they were found more in the cerebral white matter than in the cortex of the human brain (KIYOTA, 1956). On the other hand, it appears that the soluble proteins are chiefly found in nerve cell bodies which form the main part of the cortex. This could explain the higher hydrophilic property of the cortex as compared with that of white matter.

2. *Soluble protein fractions*

The results obtained by the Tiselius electrophoresis method and by fractionations with sodium sulphate (HOWE, 1921) are shown in Table 2 and in Fig. 1. Each fraction obtained by electrophoresis was provisionally described as albumin, α, β and γ-globulin, comparing them with the mobilities of serum protein fractions. This does not, however, mean that the proteins thus designated are chemically identical to the comparable fractions in serum. The fractions separated by sodium sulphate were provisionally named by the same terms as those used to identify serum protein fractions: albumin, pseudoglobulin-II, pseudoglobulin-I and euglobulin.

When tested by the Tiselius electrophoresis method, more albumin was found in the white matter than in the cortex, while the α-globulin content was greater in the cortex. The electrophoretic pattern of the thalamus was similar to that of the cerebral cortex. The content of albumin increased through white matter, medulla oblongata and spinal cord, while the α-globulin decreased. This order in human brain was almost the same as that in dog brain (ARIMA, 1958). It has been shown elsewhere (KIYOTA, 1956) that the albumin content of the cerebral hemisphere of the dog is lower, and the α-globulin content higher, at the post-natal stage of development than in the mature brain. With the maturation of cell structures accompanied by active myelination, the albumin content increases and the α-globulin content decreases. Later, the content of

TABLE 1.—WATER AND PROTEIN CONTENT OF NORMAL ADULT HUMAN BRAINS

No. of brains	Region	Water content	Dry matter	Total protein	Soluble protein	Insoluble protein	Ratio protein/ total dry matter (%)	Ratio soluble protein/total protein (%)	Ratio soluble protein/total dry matter (%)
10	Cortex	82·26 (81·77–82·60)	17·74 (17·40–18·23)	9·15 (8·91–9·43)	3·11 (2·87–3·38)	6·04 (5·71–6·38)	51·6 (49·3–53·2)	34·0 (31·0–35·9)	17·5 (16·4–18·8)
10	white matter	69·18 (68·33–69·90)	30·82 (30·10–31·67)	10·52 (9·65–11·01)	2·22 (1·97–2·57)	8·30 (7·31–8·92)	34·2 (31·8–36·0)	21·2 (18·6–24·2)	7·2 (6·3–8·2)

Age variation: 22–51 years. Figures in brackets denote extreme values. Protein values are expressed as g/100 g wet weight of tissue.

TABLE 2.—FRACTIONS OF SOLUBLE PROTEIN IN NORMAL BRAINS AS SEPARATED BY TISELIUS ELECTROPHORESIS AND BY SODIUM SULPHATE

Portions	Mean values of 10 samples	Nitrogen (mg)	Separation by Tiselius electrophoresis							Separation by sodium sulphate			
			Albumin	α-glb α_1	α-glb α_2	β-glb β_1	β-glb β_2	γ-glb γ_1	γ-glb γ_2	Albumin	p. glb II	p. glb I	eu-glb
Cortex	mg N/100 g wet wt. of tissue	497·4	67·1	54·2	127·8	59·7	84·6	46·3	57·7	74·1	15·8	19·0	388·5
	relative amount (per cent) variation	100·0	13·5 (12·3–15·2)	10·9 (9·9–11·4)	25·7 (24·3–26·3)	12·0 (11·2–13·2)	17·0 (15·3–19·7)	9·3 (7·6–12·1)	11·6 (10·5–13·4)	14·9 (14·5–15·5)	3·2 (2·9–3·5)	3·8 (3·5–4·2)	78·1 (77·2–78·8)
White matter	mg N/100 g wet wt. of tissue	355·0	94·1	31·6	50·8	44·7	61·8	41·9	30·2	55·4	10·7	25·2	263·7
	relative amounts (per cent) variation	100·0	26·5 (23·8–28·7)	8·9 (8·3–9·7)	14·3 (13·7–15·3)	12·6 (11·6–14·2)	17·4 (15·8–19·0)	11·8 (10·2–12·6)	8·5 (6·8–10·1)	15·6 (15·1–15·9)	3·0 (2·3–3·5)	7·1 (6·5–7·8)	74·3 (73·5–75·5)

Absolute amounts of nitrogen of soluble protein are expressed as mg/100 g of weight of wet tissue.

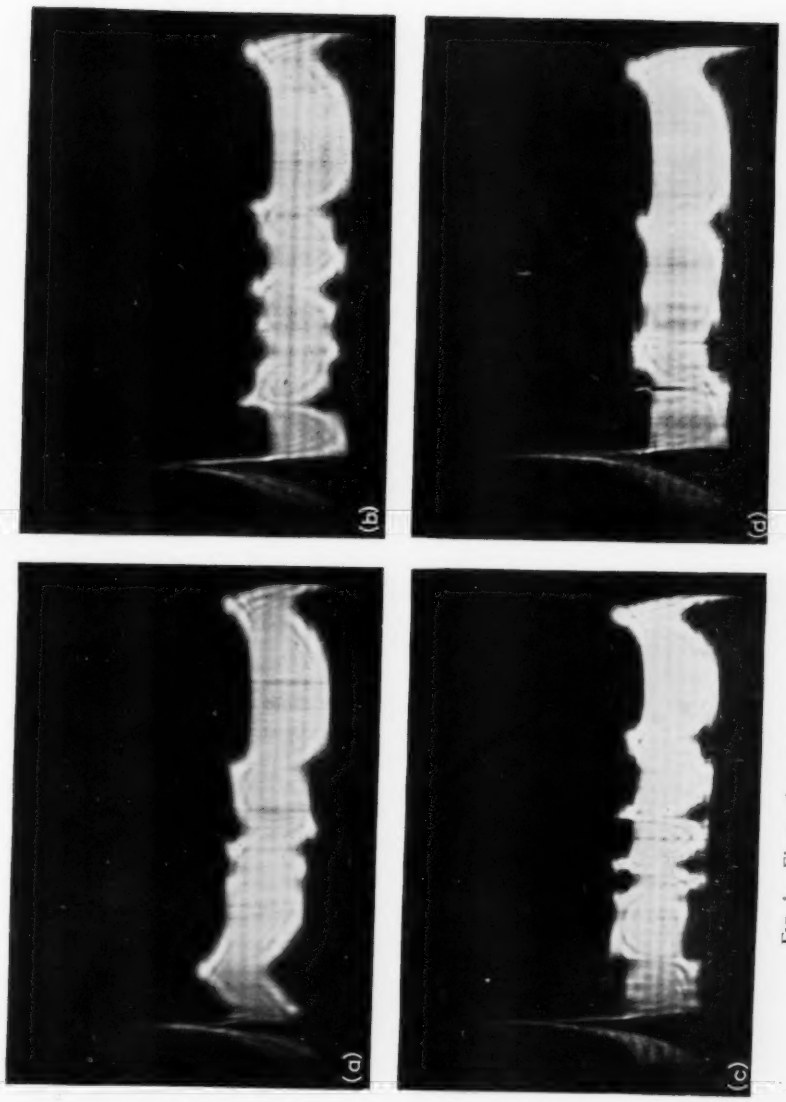

FIG. 1.—Electrophoretic patterns of different regions of the central nervous system in man: (a) cerebral cortex; (b) cerebral white matter; (c) medulla oblongata; (d) spinal cord.

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both fractions decreases until full maturity is reached. It is interesting that the relative amounts of the soluble protein fractions present in the cerebral hemisphere at the immature stage after birth are nearly the same as those of the cerebral cortex at maturity.

When fractionated by sodium sulphate, the relative amount of albumin in the white matter was similar to that found in the cortex. However, the absolute amount of albumin was lower in the white matter than in the cortex. The content of pseudo-globulin-I was greater in the white matter than in the cortex. The albumin value obtained in the cortex by fractionation with sodium sulphate was slightly higher than that obtained by the Tiselius electrophoresis method, while in the white matter, the albumin value obtained by chemical fractionation was considerably lower than that obtained after electrophoresis. In other words, a part of the albumin in the white matter fractionated by the Tiselius electrophoresis method can be precipitated by 22·2% sodium sulphate.

It has been shown elsewhere (KIYOTA, 1956) that cerebral albumin which can be precipitated by 22·2% sodium sulphate increases with the maturation of cell structures and myelination in the developing brain, and its content is greatest at the terminal stage of myelination. Moreover, it has been suggested elsewhere (KIYOTA, 1959) that the α-globulin fraction of immature dog brain is more difficult to coagulate by heating than that of mature dog brain. These protein fractions have not been chemically identified, though preliminary observations suggest that the albumin may be a glycoprotein and that the α-globulin is related to a strandin-like hydrophilic substance (unpublished data). The greater amount of α-globulin in the cerebral cortex as compared with that found in the white matter could account for the greater hydrophilic property of the cortex as compared with that of white matter.

The quantitative separation of brain proteins by paper electrophoresis has proved inferior to that obtained with the Tiselius electrophoresis. The protein values obtained have varied with the experimental conditions, e.g. protein extraction procedure, paper used for electrophoresis, the amount of extract applied, electrophoretic conditions and methods for the estimation of separated proteins (direct spectrophotometric method to measure the dyestuff attached to protein, method of dye elution and the micro-Kjeldahl method), as was clearly shown by ROBERTSON (1957). Even when certain conditions are kept strictly constant, quantitative estimates of the soluble protein fractions in human brains have shown considerable variation. Thus, it was difficult to find a significant difference in the soluble protein fraction of cerebral cortex and of white matter by paper electrophoresis. The relative amounts of the soluble protein fractions found for human brain expressed on a percentage basis were as follows: pre-albumin, 1·3; albumin, 5·2; α_1-globulin, 7·2; α_2-globulin, 16·5; β_1-globulin, 23·2; β_2-globulin, 24·6; γ-globulin, 22·0 (means taken from cortex values for seven brain samples).

It was noted that the albumin values of brains determined by paper electrophoresis were considerably lower than those found by Tiselius electrophoresis, especially for the white matter. However, the results of paper electrophoretic separations of serum under our experimental conditions have shown a slightly lower value for albumin and a slightly greater value for γ-globulin than those found by the Tiselius electrophoresis method. Paper electrophoresis is a method of separation utilizing three properties of the proteins, i.e. electrophoretic mobility, affinity for the dyestuff and

adhesion to the paper. Therefore, any change in these properties, even when the electrophoretic mobility is the same, could account for the discrepancy between results obtained by paper electrophoresis and by the Tiselius electrophoresis method. It was recently pointed out that the paper electrophoresis methods often showed results very different from those obtained by the Tiselius method in the separation of pathological serum proteins (KOBAYASHI, 1955). Also, the separation of serum proteins denatured by heating at 75°C differs when using the two electrophoretic methods (KIYOTA and FUJIYOSHI, 1958). It is well known that after standing for several days, serum shows an increased amount of protein not migrating from the origin during electrophoresis on paper.

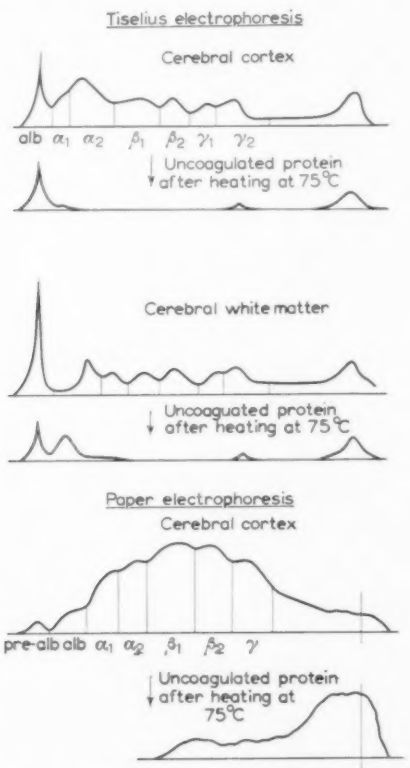
Tiselius electrophoresis
Cerebral cortex
alb α1 α2 β1 β2 γ1 γ2
Uncoagulated protein after heating at 75°C
Cerebral white matter
Uncoagulated protein after heating at 75°C
Paper electrophoresis
Cerebral cortex
pre-alb alb α1 α2 β1 β2 γ
Uncoagulated protein after heating at 75°C

FIG. 2.—Electrophoretic pattern of soluble brain proteins after heating. Tiselius electrophoresis.

Judging from these results, a significant difference between the two electrophoretic methods in the separation of the soluble brain proteins may depend, in part, on differences in the chemical nature of serum proteins and soluble proteins from grey matter and white matter. It is stressed that significant differences exist between the soluble protein fractions of grey and white matter obtained by the Tiselius electrophoresis method. On the other hand, our results using paper electrophoresis agree closely with those reported in the literature (HOFMANN and SHINKO, 1956; ROBERTSON, 1957), in which little or no difference was found between grey and white matter with

respect to soluble protein concentrations. Further work is required to clarify these points.

3. *Protein uncoagulated by heating*

Brain extracts were heated at 75° in a water-bath for 1 hr and the supernatant obtained by centrifuging at 14,500 **g** was fractionated by the Tiselius electrophoresis method. The separations were compared with those obtained with the unheated extracts (Fig. 2).

Only one albumin peak remained after heating the cortex extracts, while the albumin peak in heated extracts of white matter became smaller than that before heating, and a large new peak appeared after heating near the α-globulin position. When serum albumin is heated at 60°, the so-called C-component (SCHEER, 1941) appears near the α-globulin position, while after heating at 75°, only one large component is seen near the albumin position. These changes in the electrophoretic patterns after heating have been reported in detail elsewhere (KIYOTA and FUJIYOSHI, 1957). In short, the results show that the electrophoretic pattern of the cortex extract after heating at 75° resembles that of the serum albumin heated under the same conditions, and it is somewhat different from the pattern of the white matter extract heated under the same conditions. This fact is relevant in relation to the present results: namely, that in the cortex the amount of albumin was similar to the amount of protein not precipitated by 22·2% sodium sulphate, while in white matter the amount of albumin was greater than that amount of protein not precipitated by 22·2% sodium sulphate.

When tested by paper electrophoresis, most of the uncoagulated protein remained close to the origin. Therefore, no difference was confirmed between the cortex and the white matter extracts after heating at 75°. Brain proteins uncoagulated after heating at 75°, have the same mobilities as albumin or α-globulin in the Tiselius electrophoresis method, but they are denatured inasmuch as they can no longer migrate by electrophoresis on paper.

DISCUSSION

Several questions on the electrophoretic fractionation methods for the soluble proteins in brain have been fully discussed in another report (KIYOTA *et al.*, 1959). It was noted that the influence of the residual blood on the relative amounts of the protein fractions in the dog brain can be neglected if the removal of blood is sufficiently thorough. In the case of human material, this influence is assumed to be significant, since the blood cannot be adequately removed. However, the animal experiments (ARIMA, 1958) have clarified the question as follows: when the smaller amount of blood needed to affect the electrophoretic pattern is added to a brain extract, an increase in the γ-globulin peak is first seen, since the γ-globulin peak including the haemoglobin occupies the greatest percentage (about 84 per cent) among the peaks of the whole blood proteins in dogs. Therefore, when investigations on human materials are performed, the influence of residual blood proteins on the fractionated proteins of brain tissue is not large enough to disturb their relative amounts.

When the brain proteins are divided into two fractions, soluble and insoluble with respect to solubility in the buffer solution under certain conditions, the soluble protein is found in larger amounts in the cerebral cortex than in cerebral white matter.

In the Tiselius electrophoretic fractions of the soluble proteins, the albumin content is low and the α-globulin content is high, decreasing in the order: cerebral cortex, cerebral white matter, medulla oblongata and spinal cord. The α-globulin seems to be an important component in determining the hydrophilic property of the brain tissue and its amount is the greatest in the cerebral cortex. The albumin of the cerebral white matter is somewhat different from that of the cerebral cortex in that it contains the proteins which can be precipitated by 22·2% sodium sulphate. Also, the changes in electrophoretic patterns of the brain extract after heating differ for the cortex extract and the white matter extract. These phenomena seem to depend on differences in chemical properties between serum, cortical and white matter proteins other than their electrophoretic mobility. In paper electrophoresis, however, it was difficult to confirm the difference in the soluble protein fractions between grey and white matter.

SUMMARY

(1) A correlation between water content and protein fractions in ten brains from healthy human adults has been observed.

(2) The soluble brain proteins were fractionated by the methods of Tiselius electrophoresis, paper electorphoresis, sodium sulphate precipitation and heating. It was confirmed that there was a significant difference between cerebral cortex, cerebral white matter, medulla oblongata and spinal cord in the relative amounts of the soluble protein fractions, and also in some of their chemical properties.

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ELECTROPHORETIC PROTEIN FRACTIONS AND THE HYDROPHILIC PROPERTY OF BRAIN TISSUE—II

THE PROTEINS OF BRAINS WITH EDEMA

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THE pathogenesis of edema and of swelling of the brain are not yet understood, and the differences between these two conditions on biochemical and histological grounds remain to be clarified (REICHARDT, 1919; DE CRINIS, 1938; AMBO, 1951). The structural components mainly responsible for the retention of water in the tissue are the proteins and electrolytes. The electrolytes control the content of the 'free water' in the tissue by virtue of their osmotic pressure, while the proteins are likely to regulate the content of the 'bound water' in the tissue since they contain water of hydration resistant to osmotic pressure. It is known that the amount of water of hydration varies in the different proteins. Therefore, one might expect to find some variations in the tissue protein fractions in pathological brains showing an increased amount of tissue water. KAPS (1954) reported that albumin, which is not found in any part of the normal brain, is present in the white matter but not in the cortex of brains with edema or swelling.

The purpose of the present investigation was to classify brain edemas according to the changes in the protein fractions and to study the mechanism of the protein changes in edema. A preliminary report on this work was published previously (KIYOTA, 1957).

MATERIALS AND METHODS

The brains were removed within 6–8 hr after death from seven patients (*A*, *B*, *C*, *D*, *E*, *F* and *H*) clinically diagnosed as having edema of the brain. These brains were found to contain excessive amounts of water as measured by drying at 100°C. Some parts of the brains showed evidence of edema on histological examination, while other parts revealed no histological alteration.

The methods for extracting proteins from the brain and for electrophoretic fractionation were described previously (KIYOTA, 1959). The protein fractions obtained by electrophoresis of the brain extracts are described provisionally as albumin, α, β and γ-globulin, by comparison with the mobilities of serum protein fractions.

RESULTS

1. *Retention of water in brain tissue*

Four brain samples, *A*, *C*, *D-r* and *H* (Table 1) showed an increased water content both in the cerebral cortex and in the white matter. Four other samples, *B*, *E-d*, *E-a* and *F-a*, showed an increased water content only in the white matter. No sample showed an increased water content only in the cerebral cortex. These findings suggest that pathological retention of water in the brain appears first in the white matter. Most of the samples with an abnormal retention of water showed an increased ratio

TABLE 1.—WATER AND PROTEIN CONTENT OF BRAINS WITH EDEMA

Samples	Regions	Water	Dry matter	Total protein	Soluble protein	Insoluble protein	Protein/dry matter (%)	Soluble protein/ total protein (%)
A	cortex	84·11*	15·89*	8·41	3·00	5·41	52·9	35·7
B-l	cortex	81·95	18·05	9·06	3·05	6·01	50·2	33·7
B-r	cortex	82·19	17·81	9·00	3·11	5·89	50·5	34·6
C	cortex	83·31*	16·69*	9·15	3·81*	5·34	54·8*	41·6*
D-l	cortex	82·14	17·86	8·78	3·02	5·76	49·2	34·4
D-r	cortex	84·86*	15·14*	6·03*	3·01	3·02*	39·8*	49·9*
E-d	cortex	81·87	18·13	9·08	—	—	—	—
F-d	cortex	82·02	17·98	8·88	2·97	5·91	49·4	33·4
H	cortex	83·44*	16·56*	8·52	3·65*	4·87*	51·4	42·8*
A	white matter	73·48*	26·52*	9·49	1·86	7·63	35·8	19·6
B-l	white matter	71·32*	28·68*	10·92	3·19*	7·73	38·1*	29·2*
B-r	white matter	71·99*	28·01*	10·66	3·22*	7·44	38·1*	30·2*
C	white matter	74·89*	25·11*	—	2·08	—	—	—
D-l	white matter	67·65	32·35	10·96	1·98	8·98	33·9	18·1
D-r	white matter	80·88*	19·12*	7·24*	2·03	5·21*	37·9*	28·0*
E-d	white matter	78·76*	21·24*	7·22*	1·34*	5·88*	34·0	18·6
E-a	white matter	81·57*	18·43*	9·60	3·03*	6·57*	52·1*	31·6*
F-d	white matter	69·63	30·37	10·20	1·97	8·23	33·6	19·3
F-a	white matter	81·89*	18·11*	9·57	2·93*	6·64*	52·8*	30·6*
H	white matter	78·88*	21·12*	9·42	3·01*	6·41*	44·6*	32·0*

Water, dry matter and proteins are expressed as per cent of wet weight of tissue. *: a significant difference from control.
A: left fronto-parietal region of patient with mania (51 year old, female).
B-l and *B-r*: left and right parieto-temporal region of patient with subdural haematoma of right parietal lobe (65 year old, male).
C: left and right fronto-parietal region of patient with tumour of cerebral peduncles (48 year old, male).
D-l and *D-r*: left and right frontal lobes of patient with tumour of right hemisphere (25 year old, male).
E-d: region distant from the cancer of patient with metastatic tumour of right frontal lobe (59 year old, male).
E-a: part adjacent to the tumour of the above patient.
F-d: left fronto-parietal region of patient with tumour of right frontal lobe (54 year old, female).
F-a: part adjacent to the tumour of the above patient.
H: left and right fronto-parietal lobes of patient with collagen disease (21 year old, male).

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of soluble protein extractable with phosphate buffer (KIYOTA, 1959) to the total amount of protein in the brain tissue.

2. '*Simple edema*' *of the brain*

The cortex and white matter from sample *A* and cortex from samples *D-r* and *H*, showed only a decrease in the relative amount of dry matter due to an increased water content of the tissue (Table 1), and no change in the soluble protein fractions (Table 2). The edema in these brains can be suitably designated as '*simple edema*'.

TABLE 2.—TISELIUS ELECTROPHORETIC FRACTIONS OF SOLUBLE PROTEINS IN BRAINS WITH EDEMA

Samples	Regions	Relative amounts of soluble protein fractions (%)								
		Albumin	α-globulins		β-globulins		γ-globulins			
			α_1	α_2	β_1	β_2	γ_1	γ_2		
A	cortex	16·8	9·5	26·5	13·1	16·1	18·0			
B-l	cortex	9·3*	18·7	* 30·6	30·0		11·4			
B-r	cortex	8·8*	19·3	* 31·1	29·8		11·0			
C	cortex	31·9*	40·0		19·0		9·1			
D-l	cortex	16·3	37·6		29·6		16·5			
D-r	cortex	16·8	10·6	24·6	26·0		22·0			
F-d	cortex	16·6	11·5	24·1	13·5	16·8	7·2	10·3		
H	cortex	15·2	12·2	25·8	29·0		17·8			
A	white matter	28·7	19·8		13·8	16·4	21·3			
B-l	white matter	16·4*	16·4	* 26·3	20·8		20·1			
C	white matter	38·4*	10·4	12·0	24·6		14·6			
D-l	white matter	25·3	21·3		15·7	18·7	19·0			
D-r	white matter	40·8*	15·9*		29·0		14·3			
E-a	white matter	39·5*	24·5		28·2		7·8			
F-a	white matter	27·9	34·6*		9·6	8·1	8·8	11·0		
H	white matter	17·9*	22·6	* 28·6	7·1	9·5	6·0	8·3		

* Denotes that there was a significant difference in the albumin and α-globulin fractions compared with control values.

3. '*Complicated edema*' *of the brain*

This type of edema is characterized by abnormality of the soluble brain proteins. This was found in the cerebral white matter in 6 out of 7 cases and in the cerebral cortex in 2 out of 7 cases. The alterations in the soluble protein fractions were of two types: (a) the first type of change, found in samples *C*, *D-r* and *E-a*, was a large increase of albumin content, and (b) the second, in samples *B*, *F-a* and *H*, was an increased amount of α-globulin (Table 2).

(a) *Increased albumin content.* The pathological and clinical signs in patients with an increased albumin content in their brain proteins were as follows:

Sample *C* was taken from the right and left fronto-parietal region of a 48 year old male patient who had an intramedullary tumour, a glioma in the region of the left oculomotor nucleus in the midbrain. Clinical symptoms were as follows. The patient was admitted to the clinic after showing progressive

development of right hemiplegia and left ptosis for 3–4 months accompanied by mental dullness during the last month. Hemiplegia alternans superior was diagnosed. The patient suddenly went into a coma and died 5 days later. A fresh localized haemorrhage was found in the tumour tissue.

Sample *D-r* was taken from the right frontal lobe, far from the site of a tumour present in the right cerebral hemisphere of a 25 year old male patient. The sample examined showed no histological changes, except for a slight edema and the presence of some xanthochromic fluid. The tumour, a glioblastoma multiforme, was located subcortically in the right parietal lobe extending down to above the right lateral ventricle. Almost all of the posterior portion of the lateral ventricle was compressed, causing blockage of the ventricular space. Moreover, the tumour extended backwards to the occipital lobe and its surface was exposed in the region of the right precuneus. Cerebellum, cerebral peduncles and quadrigeminal bodies were compressed strongly downward by the tumour. A transethmoidal cephalocele was seen at the ventral surface of the right frontal lobe. Clinical symptoms were as follows. The patient developed grand mal seizures at the age of 16, which later occurred frequently in association with occasional episodes of 'dreamy states' and schizophrenic symptoms. The patient was admitted to our clinic with a 2 month history of progressive loss of vision, vomiting, headache, cerebellar ataxia and speech disturbance. Death occurred suddenly after spinal tapping was performed. Herniation of the cerebellar tonsils was found at autopsy.

Sample *E-a* was taken from the region adjacent to a tumour in the right frontal lobe of a 59 year old male patient who died of a metastatic neoplasm originating in the lung. The sample examined was edematous and contained a little xanthochromic fluid; no tumour cells were found. Clinical symptoms were as follows: the patient showed only psychotic reactions, such as depression and loss of interest, and no neurological symptoms were apparent. He was severely emaciated and died of cachexia.

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Comments. The increased amount of albumin in the white matter of these samples was not attributable to the blood remaining in the capillaries of the brain tissue, since it has been shown by ARIMA (1958) that blood mixed with brain extract effects the electrophoretic protein pattern mainly by increasing the γ-globulin peak which includes haemoglobin.

FÜNFGELD (1930) stated that gliomata accompanied by small infiltrations spreading over an extensive region of brain tissue cause a strong edematous reaction in the infiltrated normal tissue. The portions of the above three brain samples which were examined showed no tumour cell infiltration. Therefore the increased albumin found was not due to the presence of tumour cells. However, it is conceivable that an abnormal metabolic product of the tumour cell stimulated the normal tissue in the neighbourhood of the tumour, causing a change in permeability and hence allowing the diffusion of serum proteins into the tissue (PETTE, 1938; ZÜLCH, 1951; KAPS, 1954). ZÜLCH (1951) stressed this possibility, regarding it as an allergic reaction. Moreover, he grouped brain edema into two types, the first a pericapillary edema characterized by a decreased protein content, and the second a perivenal edema in which protein from the serum entered the tissue and was seen to be strongly stained on histological examination. When the serum proteins migrate into the tissue, the albumin seems to be the most mobile, since it has the smallest molecular weight and contains more water of hydration than the other serum proteins.

The fact that pressure on the brain-stem was a common feature in cases *C* and *D*, suggests a lesion of a regulatory centre for protein metabolism in the brain stem. YOSHIDA *et al.* (1953) stated that a decrease of serum albumin and an increase of serum globulin resulted from a lesion of such a centre for protein metabolism. Therefore the possibility cannot be excluded that the migration of serum proteins into the tissue is caused by a central action. From the above considerations, it appears that some stimulation, i.e. mechanical, toxic or central, etc., of the normal tissue by the tumour is likely to cause a change in permeability and hence allow an increased migration of

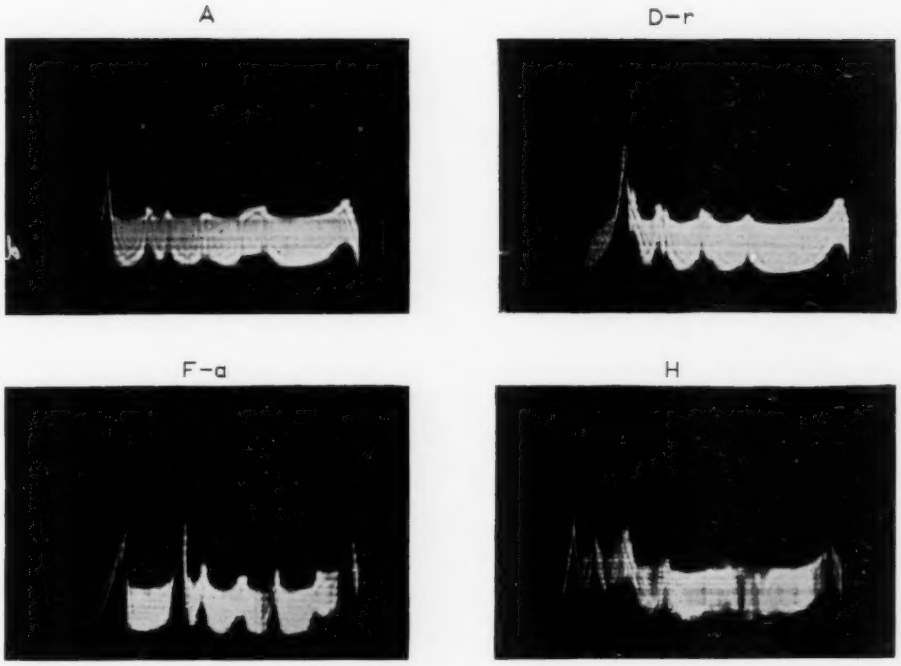

FIG. 1.—Electrophoretic pattern of soluble proteins in brains with edema. *A*: white matter of brain with 'simple edema', *D-r*, *F-a* and *H*: white matter of brains with "complicated edema". *A* shows a normal pattern of soluble proteins, *D-r* shows a large increase of albumin content; *F-a* and *H* show a large increase of α-globulin content.

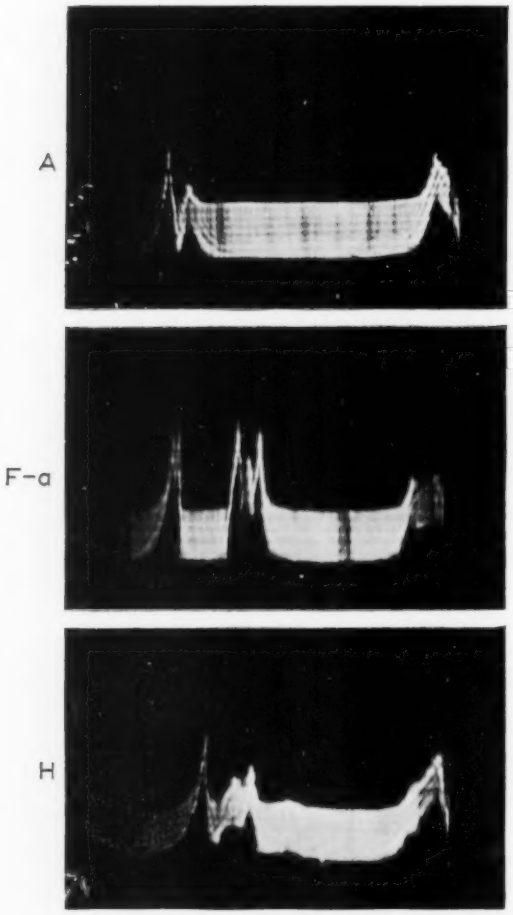

FIG. 2.—Proteins not coagulated by heating the white matter extracts. Ratios of protein not coagulated after heating to the total amount of soluble proteins before heating: *A*, 36·2 per cent; *F-a*, 71·2 per cent; *H* 69·5 per cent (these values were determined by Kjeldahl's method).

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serum albumin into the tissue. There is no conclusive evidence that the albumin fraction found to be increased in brain tissue is identical to the serum albumin. However, the results obtained on samples *D-l* and *D-r* (Table 3) suggest this explanation. The amount of albumin fractionated by the Tiselius electrophoresis method

TABLE 3.—PRECIPITATION OF BRAIN ALBUMINS BY SODIUM SULPHATE

Sample (Cerebral white matter)	Albumin fractionated by the Tiselius electrophoresis (%)	Protein not precipitated by 22·2% sodium sulphate (%)	Difference (%)
D-l	25·3	15·7	9·6
D-r	40·8	31·5	9·3

agreed with the amount of protein not precipitated by 22·2% (w/v) sodium sulphate both in sample *D-l*, which had a normal albumin content and also in sample *D-r* which contained much more albumin in the white matter. In other words, this sample showed an increased amount of a protein similar to serum albumin, both in its electrophoretic mobility, and in its solubility in 22·2% sodium sulphate. A model experiment on this point gave the result expected (ARIMA, 1958): when a known quantity of serum albumin was mixed into the brain extract, the increased amount of albumin obtained by the Tiselius method was almost the same as the increased amount of protein not precipitated by 22·2% sodium sulphate. These results indicate that the increased amount of albumin in the brain samples was probably derived from the serum albumin fraction.

(b) *Increased α-globulin content.* The pathological and clinical signs in the patients whose brains protein were characterized by an increased α-globulin level were as follows:

Sample *B* was taken from the left and right parieto-temporal region of a 65 year old male patient with a subdural haematoma on the right parietal lobe. The patient suddenly went into a coma and died 4 days later. The regions examined showed no histological change.

Sample *F-a* was taken from a region adjacent to a glioblastoma present in the brain of a 54 year old female patient and located in the subcortical region of the right frontal lobe. The region examined was strongly edematous, but no tumour cell infiltration and no other histological changes were found. The psychiatric syndrome consisted of a lowered intelligence, dementia, etc., and there were no apparent neurological symptoms. Death was due to cachexia.

Sample *H* was taken from the right and left fronto-parietal region of a 21 year old male patient who had an erythematodes disseminatus on his face and thorax, and died after frequent convulsive seizures. The volume of the brain was generally increased and it showed no histological change, but it contained a great deal of hexosamine on analysis.

In the white matter of sample *B*, a decrease in albumin and a relative increase in α-globulin were found, although the absolute amount of albumin was within the normal range and the absolute amount of α-globulin was greatly increased as a result of an increase of the total amount of soluble protein. In sample *F-a*, the absolute amount of albumin was slightly increased and the α-globulin showed a large increase both in relative and absolute content. In sample *H* the absolute amount of albumin

in the white matter was normal, while the α-globulin values were increased both on a relative and absolute basis.

Comments. In these brain samples the relative increase of α-globulin was not attributable to a decrease of albumin, since the absolute amount of albumin was not greatly changed. Therefore, a migration of albumin from the tissue into the vascular system can also be excluded for these samples. It is unlikely that the serum globulin would migrate into the tissue before the serum albumin, as the albumin is the most mobile serum protein fraction. The possibility exists, as was implied by JACOB (1940), that serum components migrate into the tissue and cause a secondary denaturation of the tissue proteins. However, no histological changes such as the edematous necrosis described by this author were found in the brain samples we have examined. The protein changes in our samples suggest a partial denaturation such as might precede the stage of edematous necrosis. In the case of a brain containing a tumour, it is conceivable that the tumour tissue itself might cause an alteration of the proteins in the adjacent normal tissue.

The increased amount of α-globulin found in sample *H* is probably related to a compound containing hexosamine, since much hexosamine was found in this preparation. Increased amounts of such hydrophilic components could account for the swelling of the brain and the convulsions observed in this particular case. In short, the mechanism for the second type of alteration in the protein fractions of the brain is not always the same; but it is likely that this alteration depends on the denaturation of the tissue protein itself and not on the migration of serum globulin into the tissue.

4. *Changes in the soluble protein fraction of the cortex in relation to disturbance of consciousness*

KAPS (1954) reported that albumin values were high in the cerebral white matter, though not in the cortex, in the neighbourhood of tumours. Complicated edema is certainly more frequent in the white matter than in the cortex. Only two brains were found in which complicated edema was observed in the cortex. Sample *B* showed a slight decrease of albumin and a large increase of α-globulin, while sample *C* showed a greatly increased amount of albumin. It was of interest that the clinical symptoms in these two patients were different from those of the other cases examined. Both died after 4–5 days loss of consciousness which was suddenly caused by an acute haemorrhage in the brain (subdural hematoma in sample *B* and haemorrhage localized in the tumour tissue in sample *C*).

This suggests that the severe disturbances of consciousness were not induced by complicated edema in the cerebral white matter, but by its extension over a wide region of the cortex. It has been shown elsewhere (KIYOTA *et al.*, 1958) that there is a slight decrease in the albumin content or in the water content of the cortex during narcosis in dogs, but not in the white matter. Two types of alteration of the cortical proteins during loss of consciousness might therefore be described. The first consists of an increase and the second, of a decrease in water or albumin content. It may be noted that the first alteration is characteristically associated with lowered convulsion thresholds. These observations suggest that a disturbance of consciousness may be caused by a change in cortical composition which is independent of a lesion of Economo's centre of consciousness or the reticular formation of the brain stem (MAGOUN, 1952).

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5. *Protein uncoagulated by heating*

The amount of protein in the buffer extract of brain uncoagulated at 75° was determined by Kjeldahl's method and protein fractionation was carried out by Tiselius electrophoresis. The heat-resistant proteins in the cortex and white matter of sample *A* and in the cortex of samples *D-r* and *H* were identical to those found in controls with respect to quantity and electrophoretic pattern. On the other hand the white matter in samples *D-r*, *E-a*, *F-a*, *H* contained predominantly heat-resistant proteins, which were localized mainly in the albumin and α-globulin positions when fractionated by the Tiselius electrophoresis method (Fig. 1).

The amount of heat-resistant protein in the cerebral hemispheres of the dog has been shown elsewhere (KIYOTA, 1959) to be greater in the young than in the adult animals and to play an important part in the retention of water in the tissue. It may be noted that the amount of heat-resistant protein of high water content was greater in the brains with complicated edema than in the brains with simple edema.

CONCLUSIONS

In pathological conditions in which water is retained in the brain, it probably appears first in the cerebral white matter and then diffuses into the cortex, where it is associated with a disturbance of consciousness or convulsive seizures.

Brain edema can be divided into two types: a 'simple edema' and a 'complicated edema'. In the former, the soluble proteins give a normal electrophoretic pattern, while in the latter they give an abnormal pattern showing an increase of albumin or of α-globulin. A large increase of albumin is presumed to be caused by the migration of serum albumin into the tissue, while an increase of α-globulin seemed to be induced by a denaturation of the tissue proteins. The mechanism of the denaturation varies according to the pathological factors involved; but the retention of water in the cerebral white matter (simple edema) might lead in time to an alteration of the tissue proteins (complicated edema). In most cases the complicated edema was confined to the cerebral white matter, and its spreading over the cortex was observed in rare cases where severe disturbances or consciousness were present.

SUMMARY

(1) Brain edema could be divided into two types, simple edema, which showed normal protein fractions, and complicated edema, showed abnormal protein fractions. The latter could also be divided into two types due to (a) migration of serum albumin into the brain tissue and due to (b) denaturation of the tissue proteins themselves.

(2) The localization of the edema in the brain and its relation to the clinical symptoms are discussed.

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EFFECT OF CONVULSANT TREATMENT ON THE 5-HYDROXYTRYPTAMINE CONTENT OF BRAIN AND OTHER TISSUES OF THE RAT

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IN communications given at international congresses and in several papers, GARATTINI and co-workers discussed the changes in the 5-hydroxytryptamine (5-HT) content of the brain and the gastro-intestinal tract following convulsant treatments. (GARATTINI and VALZELLI, 1956, 1957; FRESIA, GENOVESE, VALSECCHI and VALZELLI, 1957; JORI, VALSECCHI and VALZELLI, 1957; GARATTINI, 1958). The experiments were carried out on different animal species, but the bulk of the results concern the rat. These research workers found that both after electroshock and after leptazol convulsions, there was a rapid and conspicuous increase of brain 5-HT (up to 200–300 per cent after 10 min) with a correspondingly diminished intestinal 5-HT (about 60 per cent decrease after 10 min). Similar data have been apparently obtained both with spectrophotometric methods (UDENFRIEND, WEISSBACH and CLARK, 1955) and with biological methods (rat colon and oestrous rat uterus).

The results of GARATTINI and co-workers are at variance with those obtained by GREEN, PAASONEN and GIARMAN (1957) in man (no change of blood 5-HT content after electroshock could be observed), and by BONNYCASTLE, GIARMAN and PAASONEN (1957) who were unable to detect any change in the 5-HT level of the brain, following electroshock or administration of convulsant drugs, while succeeding in demonstrating considerable variations of brain 5-HT after anticonvulsant treatment.

Since preliminary results obtained in our laboratory some time ago were similarly at variance with those of GARATTINI and co-workers, we decided to examine more thoroughly the influence of electroshock and leptazol on the 5-HT level of several rat tissues including brain and gastro-intestinal tract.

METHODS

Convulsant treatments

Convulsant treatment was carried out on twenty-two groups of from four to eight animals each. Rats of both sexes weighing 150–300 g were used. In every experiment, control and treated animals were of the same weight and came from the same breed.

Electroshock was carried out as follows: 125 V and 130 MA for 0·2 sec. The animals were killed 10, 30, 60 and 120 min after the application of the current.

As regards leptazol, we found, in agreement with other research workers (HILDEBRANDT, 1937; FARRIS and GRIFFITH, 1949), that the intraperitoneal dose of 80–90 mg/kg used by GARATTINI and co-workers is lethal for 50–100 per cent of the animals within 15–20 min. The small number of rats treated by us with such a dose was moribund when killed. In order to obtain the survival of all animals, together with the appearance of a typical convulsive seizure, we reduced the dose to 60 mg/kg (intraperitoneal route). With a dose of 50 mg/kg only 25 per cent of rats showed typical convulsions. The animals were killed by decapitation at varying intervals after the leptazol injections.

Preparation of acetone extracts

The following tissues were examined: blood, spleen, gastro-intestinal tract, brain, lungs and ears. The first four tissues contain 5-HT in every mammalian species, the last two are rich in 5-HT only in rats and mice, as a consequence of the presence of a large amount of 5-HT in the mast cells of these animals.

Blood was kept for 2–3 hr at room temperature, then for about 12–15 hr at +4°C. The serum was separated by centrifugation and then treated with 4 vols. of acetone. After standing overnight in a refrigerator the liquid was filtered and the clear filtrate used for bioassay. The gastro-intestinal tract was opened longitudinally, washed with tap water and carefully blotted with filter paper. After this, it was minced with scissors and extracted with 4 parts (w/v) of acetone for 24 hr and then, after decanting the liquid, re-extracted with 3 to 4 parts of 80 per cent acetone for another 24 hr. The combined filtrates were stored in the refrigerator until used. All other tissues were treated in the same way as the gastro-intestinal tract. No washing, of course, was necessary.

Before the bioassay acetone was removed by evaporation under reduced pressure or, if small quantities were used, by rapid evaporation in a boiling water bath under an air stream. The remaining aqueous liquid was brought to the desired volume with physiological saline.

Treatment with 80 per cent acetone was found in our laboratory to assure a complete extraction of 5-HT from tissues. The disadvantage presented by the partial co-extraction of substance P (present only in the gastro-intestinal tract and in the brain) is widely compensated by the complete extraction of 5-HT. This cannot be obtained with 95 per cent acetone, as recommended by AMIN, CRAWFORD and GADDUM (1954). Moreover, CORREALE (1958) has shown that under our experimental conditions substance P can cause, in the 5-HT estimation, an error in excess of barely 5 per cent for the gastro-intestinal tract and of 20 per cent for the brain. At any rate, the degree of interference of substance P with the brain 5-HT estimation, was checked by us with BOL (2-brom-D-lysergic acid diethylamide): it was found that no more than 15 per cent of the original activity of our brain extracts, remained after addition to the nutrient liquid, of 0·02 μg/ml BOL.

Bioassay of 5-HT

The extracts were assayed on the atropinized oestrous uterus of ovariectomized rats, according to the method used in our laboratory (ERSPAMER, 1940; 1954). The response of the uterus preparation was recorded for 3–4 min, and the time interval between doses was 2–3 min. Although all aspects of the uterus response were considered during the assay, particular emphasis was laid on sustained contraction, and not only on the height of this contraction but also how well it was maintained. The standard was 5-HT creatinine sulphate (Farmitalia S.p.A., Milan): the values were, however, expressed as 5-HT base.

RESULTS

Electroshock experiments

Results obtained in two different electroshock experiments are summarized in Tables 1 and 2. Each value refers to the pool of tissues obtained from four rats. The following conclusions may be drawn from the tabulated data:

(1) Brain 5-HT levels seem, on the whole, to be slightly higher (up to 20–30 per cent) in animals treated with electroshock than in controls. The largest amount found in these animals was 0·40 μg/g. It should be noted, however, that a value of this magnitude was encountered even in a control group and that, independently of the present investigation, it must be considered to be within the normal range for 5-HT in rat brain.

(2) The 5-HT in the gastro-intestinal tract does not present significant changes at any time after electroshock. The fluctuations in the 5-HT content are always within the normal range.

(3) There are no changes, following electroshock, in the 5-HT content of spleen, lungs, ears and serum. With regard to serum our negative results are in full accordance with those of GREEN *et al.* (1957).

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TABLE 1.—5-HYDROXYTRYPTAMINE CONTENT (μg/g OR μg/ml) OF SOME TISSUES OF RATS TREATED WITH ELECTROSHOCK
(First experiment)

Tissue	Control animals	Treated animals			
		Time after electroshock			
		10 min	30 min	60 min	120 min
Brain	0·25 0·38	0·33	0·38	0·40	0·38
Gastro-intestinal tract	1·50 1·50	1·20	1·90	1·20	2·30
Serum	0·38 0·51	0·38	0·43	0·30	0·41
Spleen	1·20 1·30	1·60	1·35	0·86	1·03
Lung	1·20 0·90	0·90	1·20	0·90	—
Ear	0·80 0·70	0·40	0·83	0·72	0·56

TABLE 2.—5-HYDROXYTRYPTAMINE CONTENT (μg/g OR μg/ml) OF SOME TISSUES OF RATS TREATED WITH ELECTROSHOCK
(Second experiment)

Tissue	Control animals	Treated animals	
		Time after electroshock	
		10 min	60 min
Brain	0·23 0·27	0·34 0·38	0·34 0·29
Small intestine	2·40 2·20	1·75 1·80	2·06 1·93
Large intestine	3·10 3·24	2·88 3·09	3·10 4·30
Serum	0·48 0·46	0·65 0·53	0·43 0·43
Spleen	1·49 1·48	2·19 2·19	1·23 1·50
Lung	1·04 1·27	1·10 1·60	2·01 1·70
Ear	0·22 0·26	0·19 0·48	0·43 0·30

TABLE 3.—5-HYDROXYTRYPTAMINE CONTENT (μg/g OR μg/ml) OF SOME TISSUES OF RATS TREATED WITH LEPTAZOL (60 mg/kg i.p.)

Tissue	Control animals	Treated animals	
		Time after leptazol injection	
		10 min	60 min
Brain	0·24	0·33	0·34
	0·23	0·33	0·25
Small intestine	1·40	1·96	2·10
	1·70	1·90	2·10
Large intestine	4·70	4·00	7·50
	6·40	6·00	6·00
Serum	1·00	0·80	0·95
	1·00	0·84	0·65
Spleen	1·40	1·60	1·10
	1·10	1·30	1·70
Lung	1·10	2·00	2·50
	1·50	2·10	2·80
Ear	1·30	1·30	1·30
	—	1·13	—

TABLE 4.—5-HYDROXYTRYPTAMINE CONTENT (μg/g OR μg/ml) OF SOME TISSUES OF RATS TREATED WITH LEPTAZOL (80 mg/kg i.p.)

Tissue	Control animals	Treated animals
		Time after leptazol injection 15–20 min
Brain	0·28	0·38
Gastro-intestinal tract	1·80	2·70
Serum	0·60	0·77
Spleen	1·50	1·80
Lung	1·30	1·30
Ear	0·86	0·86

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Experiments with leptazol

Results obtained in rats treated with 60 mg/kg and 80 mg/kg leptazol intraperitoneally are shown in Tables 3 and 4, respectively. The conclusions which may be drawn from the tabulated data are quite similar to those reached in the preceding paragraph. As with electroshock seizures, leptazol convulsions also failed to produce any important change in the 5-HT content of the examined tissues. Especially in the case of the gastro-intestinal tract, the 5-HT level did not present the striking reduction found by GARATTINI and co-workers; on the contrary, if any change was present, this was in the direction of an increase.

DISCUSSION

In our experiments neither electroshock nor leptazol convulsions produced any important change in the 5-HT content of the rat tissues examined. As already pointed out, our negative results are at variance with those of GARATTINI and co-workers but in full accordance with those of GREEN *et al.* (1957) and of BONNYCASTLE *et al.* (1957).

It is rather difficult to find an acceptable explanation for these major discrepancies in experimental results. It may be observed, however, that some of the values obtained by GARATTINI and co-workers are unusual, as they are exceedingly low (encephalic normal 5-HT level as estimated by biological method = 0·013 μg/g) or exceedingly high (encephalic normal 5-HT level as estimated by a spectrophotometric method = 0·97 μg/g) when compared with the values found by other workers.

It should be added that the use, by GARATTINI and co-workers, of the spectrophotometric method of UDENFRIEND *et al.* (1955) in the estimation of the 5-HT content of rat tissues, and especially of brain, is rather astonishing since the investigators who have proposed this method, claim in their paper that assay by ultraviolet absorption is only applicable 'to the determination of 0·04 to 0·4 μmole of 5-HT in as much as 500 mg tissue' and that 'at present the spectrophotometric methods are best suited to metabolic studies which involve the assay of 5-HT in tissues to which it or its precursor have been added in relatively large amounts'.

Now, 0·04 μmole means 7 μg of 5-HT per 0·5 g fresh tissue. But 7 μg is the quantity of 5-HT which, according to GARATTINI and VALZELLI (1957), would be present in 7 g of normal brain (= 5–6 rats!).

The present negative results are, of course, valid only for a single convulsant treatment; however, they raise some doubt about the possibility of obtaining different results with a series of electroshocks or other convulsant treatment.

SUMMARY

The effect of convulsive seizures produced by electroshock or leptazol injections on the 5-HT content of brain and other tissues of the rat was investigated.

The brain 5-HT level of rats subjected to convulsant treatment appeared to be slightly higher (up to 20–30 per cent) than that of control animals.

The 5-HT content of other tissues and blood did not show any significant change.

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MICRODETERMINATION OF FREE AND ESTERIFIED CHOLESTEROL IN CEREBROSPINAL FLUID

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IN order to study the cholesterol levels in the cerebrospinal fluid (CSF) of patients with demyelinating and other neurologic disorders, an accurate microassay was devised for the extraction, separation, identification and determination of free and esterified cholesterol. The micromethod is based on an extraction procedure, ROBOZ *et al.*, (1958), separation by silicic acid column chromatography, FILLERUP and MEAD (1953), WYCOFF and PARSONS (1957), and colorimetric determination by H_2SO_4–$FeCl_3$ reagent, ZLATKIS, ZAK and BOYLE (1953), MACINTYRE and RALSTON (1954), ROSENTHAL, PFLUKE and SALVATORE, (1957). SELBACH and TRAPPE (1944) studied the levels of free and esterified cholesterol in CSF specimens from patients with psychiatric and neurologic diseases using an aluminium oxide column. Aluminium oxide is reported to hydrolyse esters TRAPPE (1940), FILLERUP and MEAD (1953). This difficulty was not encountered by HESS (1947) in previous work in blood. ROBOZ *et al.* (1958) used aluminium oxide to separate free and esterified cholesterol in CSF. This method requires large amounts of aluminium oxide and eluents. It was found that silicic acid could be employed for the same purpose. It has the advantage that only a small amount is required for the column and the volume of eluants is greatly reduced. No hydrolysis of esterified cholesterol was detected. The free and esterified cholesterol in CSF were identified by paper chromatography. The method has been applied to the analysis of CSF of patients with non-neurologic disorders.

MATERIALS AND METHODS

Reagents

Silicic acid—100 mesh.

Petroleum ether purified by several treatments with concentrated sulphuric acid followed by washing with water. After the water was removed with $CaCl_2$, the petroleum ether was passed through a silicic acid column and distilled. The fraction boiling between 40–60° C was used.

Chloroform–petroleum ether (1 : 1, v/v).

Ethyl acetate–petroleum ether (1 : 1, v/v).

Chloroform–methanol (2 : 1, v/v).

$FeCl_3 \cdot 6H_2O$ stock solution, 2·5% in 85% phosphoric acid.

$FeCl_3 \cdot 6H_2O$ colour reagent, 8 ml of the stock reagent in 92 ml of concentrated H_2SO_4.

Phosphotungstic acid solution 10% in absolute ethanol.

PROCEDURE

(a) *Extraction*

CSF (10 ml), obtained by lumbar puncture, are placed in a glass-stoppered test tube and 20 ml of the chloroform–methanol mixture are added. The tube is shaken well, the pressure is released,

and it is placed in a water bath at 80-85° C for a few seconds. Immersion in the water bath, shaking and release of pressure is continued until the mixture becomes warm enough to form an emulsion.

The emulsion is centrifuged for 10 min at 500 g at 10°C and the upper phase is removed by suction. The tube is washed around the surface of the solution with 2 ml of methanol to incorporate the remaining water. The extract is filtered through a fat-free Schleicher and Schuell (S & S) 'shark-skin' paper into a second glass-stoppered test tube and the first tube as well as the filter paper are rinsed with 2–3 ml of the above-solvent mixture.

Water (4 ml) is added to the filtrate and the mixture is shaken, centrifuged, and the upper phase is removed as before. After the addition of 2 ml of methanol, the solution is evaporated to dryness at 45–50° under a stream of nitrogen.

(b) *Column chromatography*

A chromatographic column is prepared, a tube 7 mm o.d. and 20 cm long, constricted at the lower end, being used. A cotton plug is placed at the constricted area and 1 g of silicic acid is added. The column is pre-wet with 2 ml of petroleum ether and packed by applying air pressure.

The dried extract is transferred to the column with three 2-ml portions of petroleum ether. The solvent, as well as the eluents, are forced through the column by light air pressure at a flow rate less than one drop per second. Wide bottom test tubes are used for collecting the eluents. The cholesterol ester is eluted first by the addition of 5 ml chloroform–petroleum ether (1 : 1 v/v). The eluate (5 ml) is collected in a test tube. Then the free cholesterol is eluted by 4 ml of ethyl acetate–petroleum ether (1 : 1) v/v; the eluate is forced through until flow ceases, and collected in a separate test tube. The solvents are evaporated to dryness at 45–50° under a stream of nitrogen.

(c) *Colorimetric determination*

To each tube containing the free and esterified cholesterol fractions, 3 ml of glacial acetate are added and the tubes are placed in a water bath at 70°. After 5 min the tubes are removed from the water bath and immediately 2 ml $FeCl_3$ colour reagent are added to each. The contents are mixed thoroughly by gentle, uniform agitation and the colour, which develops after standing at room temperature for 20 min, is read on a Bausch and Lomb Spectronic 20 Colorimeter at 560 mμ.

(d) *Paper chromatography*

Free and esterified cholesterol are extracted from CSF and separated on a silicic acid column as described in the above procedure. The dried fractions of cholesterol from CSF as well as prepared standards are dissolved in petroleum ether, applied on S & S paper and chromatographed according to the procedure of HOUSEN and DAM (1957). The chromatograms are developed for 25 min at 10° and the spots are made visible by spraying with phosphotungstic acid solution followed by drying at 100° for 3 min.

RESULTS

Standard solutions of cholesterol containing from 20 to 100 μg were used to prepare a calibration curve. In this concentration range, the colour obtained obeyed Beer's law (Table 1).

Recovery experiments were run on standard solutions of cholesterol and cholesterol ester. Cholesteryl stearate was prepared by the method of PAGE and RUDY (1930) and recrystallized from ether and alcohol. The m.p. was 78–82° in agreement with that reported. By comparing the colour equivalent produced by purified cholesteryl stearate with pure commercial samples of cholesterol and cholesteryl acetate, good agreement was found under the conditions of colour development described in the procedure (Table 1).

Recovery of cholesterol, cholesteryl palmitate, and cholesteryl stearate standards through the silicic acid column, as free compounds and mixtures, was studied and essentially quantitative results were obtained as shown in Tables 2 and 3. Cholesteryl

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TABLE 1.—COMPARISON OF COLOUR EQUIVALENTS OF FREE AND ESTERIFIED CHOLESTEROLS

Amount (μg)	Cholesterol		
	Free O.D.	Acetate O.D.	Stearate O.D.
20	0·134	0·136	0·132
40	0·260	0·262	0·264
60	0·400	0·412	0·410
80	0·540	0·550	0·530
100	0·680	0·670	0·640

TABLE 2.—RECOVERY OF FREE AND OF CHOLESTERYL STEARATE AND PALMITATE AFTER SILICIC ACID CHROMATOGRAPHY

Compound	Added (μg)	Found (μg)	Recovery (%)
Free cholesterol	30	30·6	102
Free cholesterol	50	49·5	99
Cholesteryl palmitate*	19·5	19·3	99
Chloesteryl palmitate	48·3	46·6	96·5
Cholesteryl stearate*	19·3	19·0	98·5
Cholesteryl stearate	48·5	46·6	96·5

* Calculated as free cholesterol.

TABLE 3.—RECOVERY OF FREE AND STEARYL CHOLESTEROL MIXTURES AFTER SILICIC ACID COLUMN CHROMATOGRAPHY

Mixture of free and stearyl cholesterol* (μg of each added)	Free found	% Recovery	Ester found*	% Recovery
40	40	100	40	100
40	40	100	37	92·5
60	60	100	55	91·7
60	58·5	97·5	56	93·4

* Calculated as free cholesterol.

palmitate* was purified by running it through a silicic acid column. Its recovery was studied through a second column.

In order to study the efficiency of the extraction procedure, as well as the quantitative separation of free and esterified cholesterol by the silicic acid column, recoveries

* Obtained from Dr. C. R. Treadwell of George Washington University Medical School, Washington, D.C.

were performed by addition of known amounts of standards to CSF followed by extraction, separation on silicic acid, and colorimetric determination. The recoveries ranged from 92–100 per cent as shown in Table 4.

TABLE 4.—RECOVERY OF FREE AND STEARYL CHOLESTEROL MIXTURE ADDED TO 10 ML OF CSF

Sample	Free found (μg/10 ml)	% Recovery	Ester found (μg/10 ml)	% Recovery
CSF 1	18·5	—	20	—
CSF 1 replicate	19·5	—	20	—
CSF 1 + mixture of 40 μg each*	58·0	99·9	57·5	96·8
CSF 1 + mixture of 40 replicate	57·5	96·6	55·0	91·7
CSF 2	25·0	—	24	—
CSF 2 replicate	25·0	—	24	—
CSF 2 + mixture of 40 μg each*	66·0	101·5	61	95·4
CSF 2 + mixture of 40 replicate	64·5	99·2	60	93·8
CSF 2 + mixture of 60 μg each*	83·0	97·7	80	95·0
CSF 2 + mixture of 60 replicate	81·0	95·3	79	93·8

* The cholesterol ester is calculated as free cholesterol.

The method was applied to a series of spinal fluids of 'normal' individuals† (Table 5). Determination of free and esterified cholesterol concentrations in CSF of patients with neurologic disorders and correlation with the pathology will appear in another publication.

TABLE 5.—FREE, ESTERIFIED, AND TOTAL CHOLESTEROL IN CSF (mg per 100 ml)

Sample no.	Free	Esterified	Total	% Esterified
1	0·19	0·25	0·44	57
2	0·19	0·31	0·50	62
3	0·27	0·26	0·53	49
4	0·17	0·14	0·31	45
5	0·22	0·20	0·42	48
6	0·22	0·23	0·45	51
7	0·19	0·19	0·38	50
8	0·22	0·23	0·45	51
9	0·21	0·21	0·42	50
10	0·25	0·32	0·57	56
11	0·15	0·18	0·33	55
12	0·20	0·23	0·43	54
				Average: 52 ± 4·5*

* Standard deviation.

The free and esterified fractions of cholesterol in CSF were identified by paper chromatography. The chromatographic method described by HOUSEN and DAM (1957) gave good separation when the chromatogram was developed at 10° for 25 min.

† Neurotic patients with no neurologic diseases.

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The R_F value for the free cholesterol in CSF was 0·40 compared to 0·38 for the standard; the values were 0·85 and 0·80 for esterified cholesterol in CSF and cholesteryl stearate standard, respectively.

DISCUSSION

The commercial petroleum ether gave a yellow colour when reacted with the colour reagent and interference with the true colour development resulted. Purification was necessary; the fraction that distilled between 40 and 60° gave no colour when put through the procedure.

Silicic acid was suitable for the separation of free and esterified cholesterol since it did not alter the absorbed materials and showed no reaction with the solvents. It has been successfully used by FILLERUP and MEAD (1953), WYCOFF and PARSONS (1957), and MAKHERJEE *et al.* (1957), for the separation of phospholipids, fat and cholesterol in blood.

The use of $FeCl_3$ colour reagent introduced by ZLATKIS *et al.*, (1953), MACINTYRE, and RALSTON (1954), presented difficulties in the development of colour, particularly when small quantities of cholesterol were determined. The difficulties were eliminated by preparing the stock solution in phosphoric acid as reported by ROSENTHAL *et al.* (1957). Furthermore, at room temperature the colour of cholesteryl stearate did not develop fully. By using wide bottom test tubes to obtain uniform mixing, and warming the acetic acid solution to 70° prior to the addition of the colour reagent, the colour produced was satisfactory for colorimetric determination and gave the same equivalent for free and esterified cholesterol.

The procedure can be used for routine clinical microanalysis and many samples may be determined simultaneously. It is accurate for the determination of free and esterified cholesterol in small quantities whereas the digitonide precipitation of free cholesterol and estimation of the ester may be the least accurate step of chemical analysis (ROSENTHAL and JUD, 1958).

The presence of cholesterol in CSF has been reported in the literature; however, the actual identification of free and esterified cholesterol, by paper chromatography, is shown here for the first time.

SUMMARY

A microprocedure is described for the extraction, chromatographic separation on a silicic acid column and colorimetric determination, by $FeCl_3$–H_2SO_4 reagent, of free and esterified cholesterol in CSF. The two fractions of cholesterol in CSF were identified by paper chromatography and their R_F values determined.

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EFFECTS OF FLAVINS ON THE ELECTROENCEPHALOGRAM

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FLAVIN adenine dinucleotide (FAD), which is the major flavin in the brain (YAGI, 1951*a*), may be expected to be an important hydrogen carrier in biological oxidations in the brain. In a recent investigation it was found that FAD reversed the inhibitory action of chlorpromazine on the oxidation of amino acids by a homogenate of the diencephalon (YAGI, NAGATSU and OZAWA, 1956). This suggested that FAD might be concerned in the functional activity of the brain. On the other hand, riboflavin-5′-monosulphate (FMS) was found to inhibit D-amino acid oxidase in competition with FAD (EGAMI and YAGI, 1956).

In view of these results, the effects of FMS and FAD on the electrical activity of the brain were examined. It was found, as described in a preliminary report (MURAMATSU, ANDO, NAGATSU and YAGI, 1958), that FMS and FAD had opposite effects on the electroencephalogram (EEG). In the present investigation the writers have studied the effects of various flavin derivatives on the EEG in order to obtain information on the relation between their chemical structures and their effects on the physiological activity of the brain.

MATERIALS AND METHODS

FAD was prepared by the method of YAGI, MATSUOKA, KUYAMA and TADA (1956). The purity was more than 92 per cent; other flavins, nucleic acids and metals were not present. Flavin mononucleotide (FMN) and free riboflavin (FR) were chemically synthesized. Lumiflavin and lumichrome were prepared by the photo-decomposition of FR and purified by crystallization from organic solvents (YAGI, 1952). FMS was synthesized by sulphonation of riboflavin in pyridine solution, using chlorosulphonic acid at 40°C under dry conditions. The FMS was purified by chromatography on powdered cellulose, and finally the calcium salt of FMS was obtained as yellow crystals (TAKAHASHI, YAGI and EGAMI, 1957). Adenosine monophosphate (AMP) was supplied from Schwarz Laboratories Inc. Each substance was used in $0{\cdot}25 \times 10^{-3}$ M aqueous solution.

A guinea pig was anaesthetized by intraperitoneal injection of urethane. After craniotomy, two kinds of electrode were placed on the brain; one was set on the surface of the cerebral cortex to record the electrocorticogram (ECoG), and the other was put into the thalamic region to record the deep EEG. The exact position of the deep electrode was checked by histological investigation of the brain after the experiment. The reference electrode was put on the frontal area of the cranium, and monopolar leads were taken between the reference electrode and the cortex or the thalamic region. For recording, a Grass III-D electroencephalograph was used.

When a stable EEG was obtained, 2 ml of an aqueous solution of flavin ($1{\cdot}0 \times 10^{-6}$ mole/kg of animal) were injected intra-abdominally, and the EEG changes were observed.

RESULTS

Effects of flavins and their components on the EEG

FAD. After the injection of FAD, the voltage increased in both the ECoG and in the deep EEG in the thalamus. A characteristic change was observed in the

ECoG; bursts of 10–15 cyc/sec high voltage activity in spindle form appeared periodically as shown in Fig. 1.

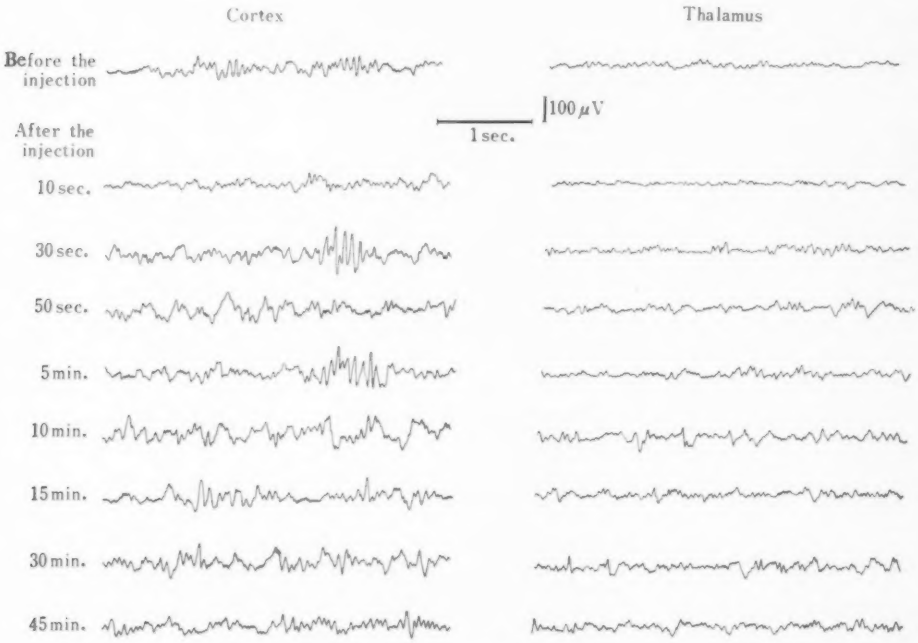

FIG. 1.—Effects of the injection of FAD on the ECoG and deep EEG from the thalamus of the guinea pig.

AMP. AMP, which is a part of the FAD molecule, was examined in the same way. AMP had a tendency to increase the voltage, but this change was less than that due to FAD, and the spindle forms were not observed.

FMN. After the injection of FMN, the voltage increased in both ECoG and deep EEG, but the increase was less than that in the case of FAD. The characteristic spindling in the case of FAD was not seen after the administration of FMN, and the frequency was not changed so markedly. Moreover, the time of duration of the changes was shorter than in the case of FAD.

FR, lumiflavin and lumichrome. After the injection of FR, lumiflavin, or lumichrome, no change was observed in either the ECoG or EEG.

FMS. After the injection of FMS, fast activity at 20–25 cyc/sec appeared in both ECoG and EEG from 30 sec after the injection, and the voltage did not increase, but slightly decreased. These changes continued for 5 min (Fig. 2). The effect of calcium ions at a concentration equivalent to that in the aqueous solution of the calcium salt of FMS was also examined; the results showed no characteristic change.

Successive administration of FAD and FMS

The changes in both ECoG and EEG after the successive administration of FAD and FMS were also examined. After administration of FAD, the changes mentioned above were observed (Fig. 3A, B). Fifteen minutes after the injection, FMS was

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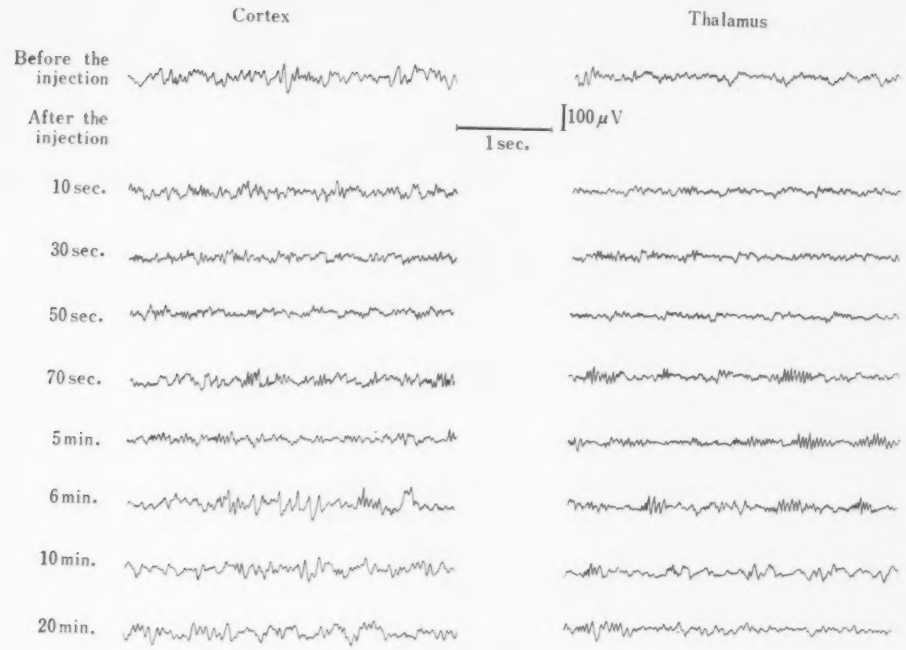

FIG. 2.—Effects of the injection of FMS on the ECoG and deep EEG from the thalamus of the guinea pig.

injected, and fast activity appeared in both ECoG and EEG (Fig. 3 B, C). Then, 15 min later, FAD was injected again, when the voltage increased gradually in the ECoG and EEG, and the frequency decreased (Fig. 3 C, D). These results were reproduced in 6 animals of each experiment.

DISCUSSION

Of the three types of flavins present *in vivo*, FAD had the most marked and characteristic effects on the EEG. This may be related to the fact that FAD is a coenzyme of important flavin enzymes not only in the brain but also in other tissues. Since neither FMN nor AMP had the same effects as FAD on the EEG, the changes provoked by FAD can be attributed to the chemical combination of FMN and AMP. FR, lumiflavin and lumichrome had no significant effect on the EEG. It therefore appears that FR itself and the *iso*alloxazine nucleus are ineffective.

On the other hand, FMS, which is the analogous compound to FMN, had the opposite effects to FAD on the EEG, and the changes due to FMS and that of FAD were competitive. A competitive action of FMS to FAD was shown also *in vitro* using the enzyme D-amino acid oxidase (EGAMI and YAGI, 1956). It is interesting to see that the antagonistic action of two flavin compounds found in a pure enzyme system was also observed in physiological phenomena such as the EEG.

It may be noted that the changes provoked by FMN were different from those produced by FMS, although the experiments carried out *in vitro* with the D-amino acid oxidase showed that both FMS and FMN inhibited oxidase activity in competition

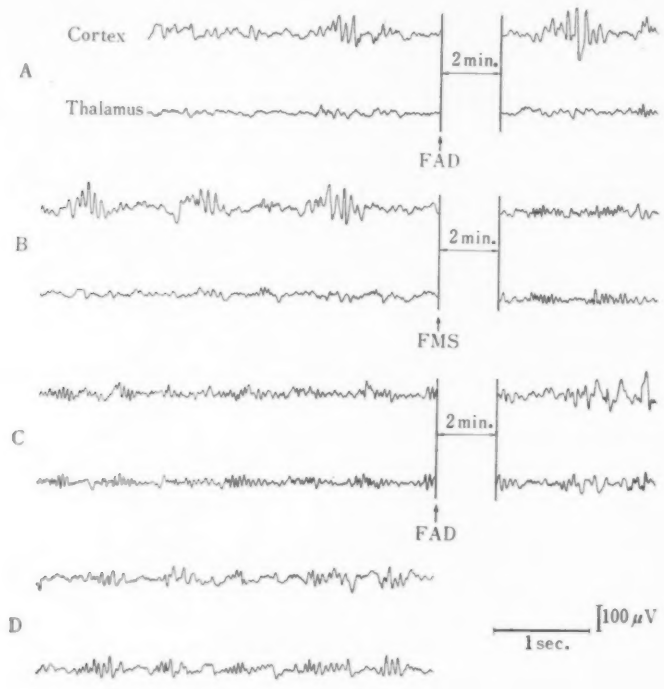

FIG. 3.—Effects of the successive injection of FAD and FMS on the ECoG and EEG of the guinea pig.

(A) ECoG and deep EEG from the thalamus before and after the administration of FAD.
(B) the effect of the administration of FMS on the continued ECoG and deep EEG of (A).
(C) the effect of the second administration of FAD on the continuation of (B).
(D) continuation of (C). The records B, C and D were continued from the previous records without interruption.

with FAD. This might be related to the fact that FMN is the biological precursor of FAD *in vivo* (YAGI, 1951*b*), while FMS is a synthetic inhibitor: the actions of FMN and FMS on the living tissue might therefore be expected to be different from those in the enzymic reaction *in vitro*.

SUMMARY

The effects of flavins on the electrocorticogram and deep electroencephalogram from the thalamus of the guinea pig were observed. FAD produced the most characteristic changes, which were an increase in voltage and the appearance of 10–15 cyc/sec high voltage activity in spindle form. FMN somewhat increased the voltage. FR, lumiflavin and lumichrome produced no significant changes in the electroencephalogram. After the injection of FMS, fast activity and a slight decrease in the voltage were observed. The effects of FAD and FMS on the electroencephalogram were opposite to each other. These results are discussed from the point of view of the enzymic action of flavins.

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GANGLIOSIDE AND ITS RELATIONSHIP TO NEURAMINIC ACID AND HEXOSAMINE IN THE BRAIN

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A RECENT paper on cerebral lipids in children included estimations of neuraminic acid in the cerebral cortex and of total hexosamine in the cerebral white matter (CUMINGS, GOODWIN, WOODWARD and CURZON, 1958). Neuraminic acid forms part of ganglioside, but neither the exact formula nor the method of estimating this latter substance has been agreed between the various groups of workers; while KLENK (1957) has employed methoxyneuraminic acid as his standard for estimating ganglioside, SVENNERHOLM (1957*a*) has used N-acetylsialic acid for the same purpose. Further, FOLCH, ARSOVE and MEATH (1951) isolated from brain a substance which they named strandin, and which contained a chromogenic group found to be akin to, or identical with, bovine sialic acid (FOLCH, MEATH and BOGOCH, 1956; LE BARON and FOLCH, 1957). However, KLENK (1957) still refers to ganglioside and neuraminic acid which he had described previously and these names will be used here, for the neuraminic acid we used as a standard was supplied by Professor E. KLENK. EDGAR (1956*a*) has suggested that the hexosamine content of a lipid extract could usefully be employed as an index of ganglioside not only in the cerebral cortex but also in the white matter, for ganglioside is known to contain galactosamine. Moreover, as gangliosides are known to contain hexoses (KLENK and LAUENSTEIN, 1953; SVENNERHOLM, 1956*a*), it is likely that the cerebrosides estimated in the brain by means of their galactose equivalent have included the hexoses that are present in ganglioside. This could be of special practical importance in diseases in which the gangliosides are increased, such as in amaurotic familial idiocy, and EDGAR (1956*b*) has discussed this extensively. This paper is an attempt to answer some of the questions raised by these problems.

MATERIALS AND METHODS

Brains were obtained at autopsy from full term infants, from children, and from adults. All brains examined were in an unfixed state, apart from one experiment concerning water-soluble hexosamine in which comparisons were made between fresh and the formalin-fixed material. The cerebral cortex and the cerebral white matter were very carefully separated and the various lipids estimated in each.

The cerebrosides and the water content were estimated as described in previous papers (CUMINGS, 1953; CUMINGS *et al.*, 1958). Neuraminic acid was estimated by the method previously employed (CUMINGS, 1957) except that the Bial's orcinol reagent was prepared according to SVENNERHOLM (1957*b*), methoxyneuraminic acid being used as the standard. Hexosamine was estimated as described previously (CUMINGS, 1957) using glucosamine hydrochloride as a standard, for it has been found that galactosamine gives the same intensity of colour as glucosamine for equivalent amounts (ELSON and MORGAN, 1933; SVENNERHOLM, 1956*b*). Dry cerebral tissue after an initial extraction by acetone was extracted with methanol–chloroform (2 : 1 v/v), and this extract was the 'lipid-soluble' fraction while the remainder of the extracted tissue was the residual fraction, the methods

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of extraction following closely those used by SVENNERHOLM (1957*a*). A water-soluble extract was obtained by following the extractive procedures of BRANTE (1957) and the quantity of hexosamine was estimated as above.

RESULTS

Tables 1 and 2 record the quantities of various lipids actually estimated in the cerebral cortex and white matter, respectively, in a group of children and adults. In addition, the ratio of neuraminic acid to lipid hexosamine is included. According to

TABLE 1.—LIPIDS ESTIMATED IN CEREBRAL CORTEX
(g/100 g dry tissue)

Age	Neuraminic acid (N.A.)	'Lipid-soluble' hexosamine (L.H.)	N.A./L.H.	Residual hexosamine	Water-soluble hexosamine	Water (%)
Full term infant	0·27	0·06	4·5	0·37	0·07	88·4
Full term infant					0·10	89·8
3 months	0·32	0·11	2·91	0·29		88·5
10 months	0·35	0·13	2·69	0·38		84·7
2 years	0·38	0·15	2·53	0·49	0·05	84·7
3 years	0·40	0·14	2·86	0·60		84·1
5 years	0·41	0·09	4·55	0·38	0·04	84·9
7 years	0·35	0·12	2·91	0·48		83·7
12 years	0·22	0·08	2·75	0·32	0·03	78·9
26 years	0·20	0·09	2·22	0·32	0·05	84·2
53 years	0·27	0·08	3·37	0·31		81·9
70 years	0·30	0·14	2·14	0·27		84·9

TABLE 2.—LIPIDS ESTIMATED IN CEREBRAL WHITE MATTER
(g/100 g dry tissue)

Age	Neuraminic Acid (N.A)	'Lipid-soluble' hexosamine (L.H)	N.A./L.H.	Residual hexosamine	Water-soluble hexosamine	Water (%)
Full term infant	0·15	0·07	2·14	0·33	0·12	85·5
Full term infant					0·11	82·7
3 months	0·29	0·10	2·9	0·61		86·8
10 months	0·10	0·05	2·0	0·32		79·7
2 years	0·07	0·04	1·75	0·35	0·02	75·7
3 years	0·08	0·05	1·6	0·24		74·9
5 years	0·08	0·03	2·66	0·26	0·03	71·2
7 years	0·07	0·05	1·4	0·25		71·3
12 years	0·09	0·03	3·0	0·19	0·01	74·8
26 years	0·05	0·02	2·5	0·21	0·01	67·9
53 years	0·04	0·02	2·0	0·23		67·4
70 years	0·04	0·04	1·0	0·23		71·7

KLENK (see EDGAR, 1956*b*) the amounts of neuraminic acid and hexosamine in ganglioside (23·4% and 8·03% respectively) are lower than those quoted by BOGOCH (1958) (30·5 and 10·8 per cent) but the ratios of these two substances are of the same order, namely 2·91 and 2·82, respectively. The ratios which are recorded here

for the cerebral cortex show that under the conditions of our experimental procedures there is a reasonable degree of agreement. However, there is less agreement for the white matter.

The amounts of ganglioside present in the white matter at almost any age greater than 3 months are small, and are in most samples not more than one quarter of those found in the cortical tissues. In the cortex the amounts of ganglioside appear to be greatest between 3 months and 5 years, although it might be noted that there appears to be some increase in later life, a fact we have observed on a number of occasions. Gangliosides in the white matter after the age of 3 months remain low, and are lowest in adult life. 'Cerebroside' value based on hexose estimations include substances other than true cerebroside, as shown by RADIN, LAVIN and BROWN (1955) and SVENNERHOLM (1956*a*). EDGAR (1956*b*) has suggested that the hexose equivalent of the ganglioside should be substracted from the galactose 'cerebroside' to obtain true values of cerebroside. In view of the differences of opinion between different workers as to the amounts of hexose in ganglioside (KLENK gives 40·3 per cent, while BOGOCH quotes 24·4 per cent) it would appear unlikely that calculations using these figures would give results of any value. From figures obtained previously (CUMINGS *et al.*, 1957) and from further values found for two infants and three adults, it is obvious that the hexose in ganglioside can in no way account for the differences found between galactose 'cerebroside' and true cerebroside.

BRANTE (1957) demonstrated in the cow and in the human another brain hexosamine fraction which was water-soluble. A few estimations have been made in some of the same material as had been used in the previous experiments, and the results are also recorded in Tables 1 and 2. It is seen that the amounts present are small and decrease with increase of age. Only a limited number of investigations were possible as it was discovered at a late stage of the experimental procedure that formalin fixed brain was useless for this determination. Much of the water-soluble hexosamine had passed into the fixative and could be estimated in the solution with only a small proportion remaining in the brain, as shown in the following figures from one representative experiment.

	Water-soluble hexosamine (mg)
Fresh cerebral cortex	64·0
Formalin-fixed cerebral cortex	5·0
Fresh white matter	16·0
Formalin fixed white matter	5·0

(All results in 100 g dry brain tissue).

It is, therefore, impossible to apply this procedure to stored material from various forms of lipidosis. However, these methods can now be applied to fresh material.

DISCUSSION

There appears to be little doubt that it is possible to estimate ganglioside reasonably accurately by either the neuraminic acid method or by means of the lipid hexo-

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samine technique, as was indicated by EDGAR (1956*b*). The former method is technically easier for the cortex, but the reverse is true for the white matter. KLENK (1957) has stated that it is almost impossible to estimate neuraminic acid accurately in white matter by his methods. This had been found by us to be true, and it was for this reason that no record of estimations of neuraminic acid in white matter were included in a previous paper (CUMINGS *et al.*, 1957). However, using the methods quoted it has now been possible to repeat all neuraminic estimations on the available material and to obtain reasonably satisfactory results. However, the presence of increased ganglioside is only of pathological significance, as far as is known, in the cortex. There is positive evidence that gangliosides are present in the white matter, as believed by both EDGAR and SVENNERHOLM, but their low level at all ages after 3 months is now established. The slightly higher figures obtained at the start of myelination may be significant. It has not yet been possible to show the site of the residual hexosamine, but it may well be present intracellularly in such cells as the astrocytes or oligodendrocytes. It increases in amount up to 3 years in the cortex. In the white matter the amount decreases with increasing age.

SUMMARY

Estimations of neuraminic acid, water soluble hexosamine, 'lipid-soluble' and 'non-lipid soluble' hexosamine have been made in cerebral cortex and white matter at various ages. It has been shown that the ganglioside content of both white and grey matter can be calculated from the amounts of both lipid hexosamine and neuraminic acid, and fairly good agreement has been obtained between the two methods. Ganglioside is present in white matter as well as in the cerebral cortex.

It has been shown that water soluble hexosamine can be estimated in fresh material only.

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EFFECT OF HYDROGEN-ION CONCENTRATION ON THE CARBOHYDRATE METABOLISM OF BRAIN TISSUE

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RELATIVELY little has been written concerning the correlation between hydrogen-ion concentration and carbohydrate metabolism of brain tissue. CANZANELLI (1939) and his colleagues studied the oxygen consumption of brain tissue from the guinea pig in horse serum at varying pH, and found that up to pH 6 the oxygen uptake increases rapidly. At a pH between 6 and 8 this increase is less pronounced: it again rises rapidly up to pH 9, and above this it shows a sharp decrease. ELLIOTT and BIRMINGHAM (1949) found that the maximum respiration of brain suspensions in different media was between pH 7 and 7·5. In the case of slices the pH actually existing in the slices may be assumed to be different from that in the medium. In their opinion it is possible that the difference in pH in slices and in the medium may represent the true physiological situation, even if the difference is physiologically not so great. BIRMINGHAM and ELLIOTT (1951) state further that in brain suspensions the optimum for anaerobic glycolysis of glucose is at about pH 7, aerobic glycolysis being greater at pH 7·3 than at pH 6·6. According to LUNDSGAARD (1933), glycolysis of surviving brain tissue was lower at pH 8·3 than at pH 7·2 or 7·3. CRAIG and BEECHER (1943) and CRAIG (1944) investigated the influence of carbon-dioxide and oxygen tension on the respiration of the cortex, medulla and retina. In respect of other tissues WARBURG and coworkers (1924, 1925) showed in carcinoma slices, as did BIRD and EVANS (1949) for bone marrow, that anaerobic glycolysis of these tissues increases from pH 6·4 to 7·4.

In connexion with the chemical analysis of neuropathological syndromes associated with alkalosis, we have investigated in some detail the correlation of pH with carbohydrate metabolism in the brain. The investigations were made at a pH between 6·8 and 7·8, in what may be considered to approach physiological conditions. An abstract of the various results of these studies has been published (DOMONKOS and HUSZÁK, 1956).

METHODS

For some experiments on cats brain slices were used: in others the brain tissue was minced with fine ophthalmological scissors. According to WARBURG's (1923) calculations, the CO_2 tension in the slices is theoretically about 3 per cent greater than in the medium. ELLIOTT and BIRMINGHAM (1949) found experimentally that the pH in the slices is to a certain extent independent of the environment. For this reason they worked with brain suspensions. In view of the disadvantages of suspension and slice techniques we thought it better for our experiments to cut up the brain tissue into small fragments with ophthalmological scissors. It was thought that with this technique more intact nervous structure could be conserved and the effect of the experimental medium would also have more influence on such small particles.

Our experiments were made with the bicarbonate–CO_2 solution of KREBS and HENSELEIT (1932) and with isotonic sodium phosphate buffer (0·066 M-Na_2HPO_4–NaH_2PO_4). In our experience the Krebs–Ringer phosphate solution was unable to maintain the desired pH values and it was not considered suitable for our experiments. We made the experiments with Krebs–Henseleit solution at

a gas tension of 1% CO_2–99% O_2 and 10% CO_2–90% O_2. At 10% CO_2 tension CO_2 was bubbled through the solution before the experiment was begun. Then a mixture of 10% CO_2–90% O_2 was blown through the incubating vessel. In the experiments made with 1% CO_2, perfusion with CO_2 was omitted and the 1% CO_2–99% O_2 gas mixture was simply blown through the incubating vessel.

The experiments with isotonic Na phosphate buffer were made at a pH between 6·8 and 7·8. In the aerobic experiments the gaseous phase was pure oxygen and in the anaerobic, pure nitrogen. The pH values were also controlled electrometrically (Methrom Swize apparatus). Our control measurements showed a shift to acid pH at the end of the experiment amounting at pH 6·8 to about 0·05, and at pH 7·8 to 0·1 and 0·2. The results were referred to the pH values obtained at the beginning of the experiment and 250 to 300 mg cortex were suspended in 2·5 ml medium at 37·5°C. The animals were killed by decapitation. Experiments were begun 15 to 20 min after killing the animals. The duration of each experiment was 45 min. The oxygen uptake was measured with a Warburg manometer. Glucose determinations were made by the method of SHAFFER and SOMOGYI (1933), lactic acid by that of BARKER and SUMMERSON (1941) or in the presence of pyruvate by that of HULLIN and NOBLE (1953); the pyruvate by the procedure 'B' of FRIEDEMAN and HAUGEN (1943), the high-energy phosphate (the phosphate hydrolysable in 7 min) by that of FISKE and SUBBAROW (1925). The sodium lactate preparation was made from calcium L-lactate using Dowex 50 ion exchange resin; sodium pyruvate was prepared by the method of LARDY, UMBREIT, BURRIS and STAUFFER (1948).

RESULTS

Table 1 presents the correlations between the CO_2 tension in the gas phase and glucose utilization. It was found that with a decrease in CO_2 tension there was an increase in the aerobic glucose utilization by the brain tissue. The results were obtained with slices and with tissue fragments.

TABLE 1.—EFFECT OF CO_2 TENSION ON THE AEROBIC CARBOHYDRATE METABOLISM OF BRAIN CORTEX SLICES AND TISSUE FRAGMENTS

(Incubated in Krebs-Henseleit solution; substrate: 6 mM-glucose)

	Glucose utilization (μmoles/g per hr)	Pyruvate formation (μmoles/g per hr)	Lactate formation (μmoles/g per hr)	High-energy phosphate (P_7) (μg/g)
Slices				
CO_2 : 10% O_2 : 90%	18·4 ± 1·7 (3)*	1·2 ± 0·4 (3)	15·3 ± 2·7 (3)	89 ± 19 (5)
CO_2 : 1% O_2 : 99%	27·7 ± 2·2 (3)	2·8 ± 0·3 (3)	29·1 ± 3·1 (3)	96 ± 15 (5)
Tissue fragments				
CO_2 : 10% O_2 : 90%	19·1 ± 2·1 (4)	1·1 ± 0·2 (4)	15·8 ± 2·1 (4)	
CO_2 : 1% O_2 : 99%	32·4 ± 2·8 (4)	4·5 ± 0·4 (4)	35·7 ± 3·4 (4)	

* Mean ± standard deviation; figures in parentheses give number of experiments.

As may be seen from Table 1 at the same CO_2 tension we observed a greater carbohydrate metabolism in the alkaline pH range (1% CO_2) with the tissue fragments than with slices. Our results seem to support the theoretically calculated values of WARBURG (1923) and the experimental results of ELLIOTT and BIRMINGHAM (1949), i.e

the pH of the slices, particularly in the alkaline region, diverges more considerably from the pH of the medium. Our experiments made with brain slices and with minced brain particles also showed that the pH effects expected of the environment are much less evident in the brain slices than in the tissue fragments. Further experiments were therefore carried out with the latter method.

To obtain more definite pH values, we employed an isotonic sodium phosphate buffer. The changes occurring in the pH range 6·8 to 7·8 were studied. Fig. 1 shows that the aerobic glucose utilization by brain tissue rises in parallel with the pH increase.

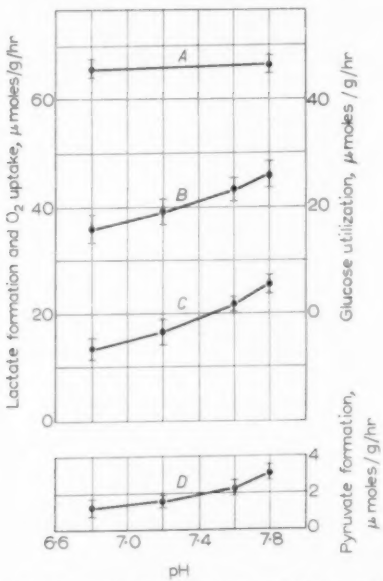

Fig. 1.—The effect of pH on the aerobic carbohydrate metabolism of brain cortex fragments. Fragments incubated under oxygen in Na phosphate buffer; substrate: 6·0 mM glucose. A: oxygen uptake, B: glucose utilization; C: lactate formation; D: pyruvate formation. The means of ten experiments and their standard deviations are shown by points (•) and short bars.

Between these two limits of pH the difference in glucose uptake is 40–45%, whereas between 7·2 and 7·6 it is 12–16%. The experiments made under anaerobic conditions led to contrary results, namely that the anaerobic utilization of the glucose diminished with a rise in pH (Fig. 2).

In similar studies on glucose utilization under aerobic conditions we also measured the production of lactic acid. The aerobic lactic acid production, as Table 1 and Fig. 1 show, increased in parallel with the rise in pH. In the course of anaerobic glycolysis the lactic acid formation also changes in parallel with the utilization of glucose; but under these conditions both undergo a decrease as the pH rises (Fig. 2).

The formation of pyruvate, both in Krebs–Henseleit and sodium phosphate buffer, varies in parallel with changes in the pH of the medium. Fig. 1 and Table 1 show that the pyruvate accumulates in parallel with an increase in pH under aerobic conditions. Hitherto, it has been thought that pyruvate occurs as an intermediary substance, in a small and relatively stable amount in a system metabolizing carbohydrate, and

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increases only when inhibiting substances are added. In the pH range of 7·2 to 7·6, we found that at pH 7·6, 35–40 per cent more pyruvate accumulated than at pH 7·2, and at pH 7·8 there was three times as much as at pH 6·8. There is no pyruvate accumulation at all during anaerobic glycolysis. (Fig. 2)

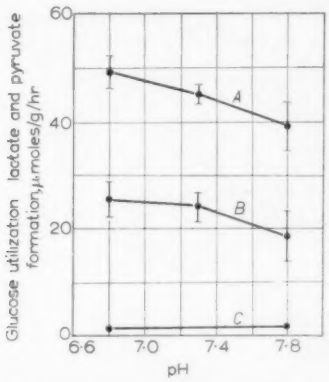

Fig. 2.—The effect of pH on the anaerobic carbohydrate metabolism of brain cortex fragments. Fragments incubated under nitrogen in Na phosphate buffer; substrate: 6·0 mM-glucose. A: lactate formation; B: glucose utilization; C: pyruvate formation. The means of five experiments and their standard deviations are shown by points (•) and short bars.

Studying the oxidative processes of brain tissue, it was found that between pH 6·8 and 7·8 there was no significant change in oxygen uptake in brain tissue (Fig. 1). We found that under aerobic conditions between pH 6·8 and 7·8 equal quantities of pyruvate and lactic acid, respectively, are metabolized. Thus a rise in pH between these limits does not diminish pyruvate or lactic acid utilization to any appreciable extent (Table 2).

TABLE 2.—THE EFFECT OF pH ON THE PYRUVATE AND LACTATE UTILIZATION OF BRAIN CORTEX FRAGMENTS

(Incubated in Na-phosphate buffer under oxygen; substrate: 3·3 mM pyruvate; 15 mM lactate)

pH	Pyruvate utilization (μmoles/g per hr)	Lactate utilization (μmoles/g per hr)
6·8	33·1 ± 2·9 (10)*	15·8 ± 1·4 (8)
7·4	34·0 ± 2·1 (4)	
7·8	33·5 ± 2·0 (10)	16·3 ± 2·2 (8)

* Mean ± standard deviation; figures in parentheses give number of experiments.

In measurements of the *in vitro* production of energy-rich phosphates (P_7), it was found that at different CO_2 tensions, i.e. at different pH values, there were no significant changes in P_7 values (Table 1).

DISCUSSION

From our results it may be concluded that the carbohydrate metabolism of brain tissue is very sensitive to changes in the pH of the medium. In these experiments we studied the metabolic changes between pH 6·8 and 7·8 and did not consider changes below pH 6·8 or above 7·8. The interval between the physiological limits of pH 7·2 to 7·6 was given the most attention. As the experimental results show, even between these relatively narrow physiological pH limits, well-defined characteristic changes occur in the glycolytic process, whereas in the oxidative system there is no measurable change.

According to the observations of BIRMINGHAM and ELLIOTT (1951) the aerobic glycolysis in brain suspensions is greater at pH 7·3 than at pH 6·6. They also stated that in the brain suspension disappearance of lactic acid—as added substrate—takes place more rapidly at pH 6·6 than at pH 7·3. In their opinion: 'This difference in rate of lactate utilization might account for part of the change with pH in net acid formation from glucose.' According to our investigation, at pH 7·8 about 40 to 45 per cent more glucose is utilized than at pH 6·8. Yet between these two pH values there was no difference in lactic acid utilization. It is our opinion that the increase in lactate production occurring with a higher pH is due to a greater glycolytic activity and not to the decreased lactic acid utilization. In the work of BIRMINGHAM and ELLIOTT (1951) experiments on glucose utilization are not to be found.

Another interesting point in our experiments was that, in parallel with an alkali rise in pH, pyruvate accumulates. Pyruvate production is a sensitive indicator of the change in pH. As in the case of lactic acid, here too we investigated whether the pyruvate accumulated at an alkaline pH, because the activity of the system oxidizing pyruvate decreased. It was established that brain tissue metabolizes pyruvate aerobically to the same extent at pH 6·8 and at 7·8. Hence it is justifiable to conclude that the pyruvate accumulation at an alkaline pH, as in the case of lactic acid, is not due to damage to oxidation system, but to an increase in glycolytic activity. In all probability the hexokinase system is responsible for the changes in glycolytic activity and the lactic acid dehydrogenase system for the accumulation of pyruvate. The intact state of the oxidation system also seems to be shown by the fact that, at pH values between 6·8 and 7·8 no measurable change could be observed in the oxygen consumption of the brain tissue or in the *in vitro* production of high-energy phosphate. It is hence an important fact that, whereas the change in hydrogen ion concentration occurring in the pH range mentioned does not affect the oxidation system, it acts, to a pronounced degree, upon the glycolytic system.

ELLIOTT and BIRMINGHAM (1949) express the opinion that under anaerobic conditions the cerebral pH depends at least in part upon the metabolic activity. On the basis of our experiments it seems reasonable to conclude that under aerobic conditions lactate formation, as well as the actual CO_2 tension, has an important role in pH regulation. Furthermore, the carbon dioxide and lactate formation retroactively influence the carbohydrate metabolism of brain tissue. A shift of the pH of the cell in an alkaline direction brings about increased lactate and pyruvate production; these substances, as acidic products, liberate carbon dioxide from the alkaline reserves of the tissue, which naturally means a shift of pH in the acidic direction. The pH shift towards acidity, however, diminishes the utilization of glucose. In this way, therefore, in carbohydrate metabolism the acidic products, as an automatic regulating system, influence the aerobic glucose utilization of the brain.

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SUMMARY

The glycolytic activity of the brain is very sensitive to changes in pH. In the range between pH 6·8 and 7·8 aerobic glucose utilization, and hence aerobic lactate and pyruvate production, increase in parallel with a rise in pH. The pyruvate produced in the course of glycolysis is an index of the pH.

The activity of the oxidative system does not alter to any appreciable extent in the pH range 6·8 to 7·8: there is also no difference in oxygen consumption, in pyruvate or lactate utilization, or in the production of high-energy phosphate. Hence the cause of the aerobic accumulation of lactate and pyruvate occurring at alkaline pH is not an impairment of the oxidation system; but it is due to an increase in glycolytic activity.

Anaerobic glycolytic activity diminishes with a rise in pH; anaerobically there is no pyruvate production.

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SPECIES DIFFERENCES IN THE DISTRIBUTION OF EXTRANEURONAL CHOLINESTERASES WITHIN THE VERTEBRATE CENTRAL NERVOUS SYSTEM

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ALTHOUGH observations on the qualitative and quantitative distribution of 'true' or acetylcholinesterase are extensive and generally consistent, there has been no systematic investigation of the phyletic distribution of 'pseudo' or butyrylcholinesterase.* The few recorded observations indicate that butyrylcholinesterase occurs not only in receptors such as the Pacinian corpuscle (HEBB and HILL, 1955), but also in the neuroglia cells and vascular endothelium in the central nervous system of the rat (KOELLE, 1952) and cat (KOELLE, 1950). The vessel walls of the frog's brain, however, exhibit no BuThChE, but only AChE activity (SHEN *et al.*, 1955). The present report is primarily concerned with the extraneuronal localization of cholinesterases within the central nervous system of several classes of vertebrates.

MATERIALS AND METHODS

Samples of brain and spinal cord from two specimens of each of the following species were examined: goldfish (*Carassius auratus*), toad (*Bufo marinus*), box turtle (*Terrapene carolina*), rooster, albino rat and cat. One specimen each of sheep, monkey (*Macaca mulatta*) and man was also examined. The samples of tissue were removed after the following treatments. The fish were placed in an aqueous solution of 0·025% w/v tricain methanesulphonate (Sandoz) until they became quiescent, whereupon they were immediately removed and dissected. Unanaesthetized rats, turtles and toads were decapitated. The cats and monkeys were anaesthetized by the intraperitoneal injection

* The following abbreviations will be used throughout this article: AChE = 'true' or acetylcholinesterase; AcThChE = 'true' or acetylthiocholinesterase, the enzyme which hydrolyses acetylthiocholine but not butyrylthiocholine; BuThChE = 'pseudo' or butyrylthiocholinesterase, the enzyme which hydrolyses butyrylthiocholine at a faster rate than acetylthiocholine; DFP = di-*iso*propylfluorophosphate.

All sections are 22 μ thick and, except where stated, were taken from the spinal cord. Photography was with transmitted light.

FIG. 1.–*Cat.* Dorsal and ventral horns. Butyrylthiocholine substrate (120 min). The neuroglia cells of the white and grey matter exhibit BuThChE activity which is especially intense in the neuroglia of the ventral horn. Here, the neuron cell bodies are surrounded by CuS deposit, but are themselves free of granules. The *substantia gelatinosa* (SG), which has very little BuThChE activity, overlies the active grey matter of the dorsal horn. ×33.

FIG. 2.–*Rat* Ventral horn. Butyrylthiocholine substrate. (90 min). The endothelium of capillaries in the ventral grey and white matter exhibits marked enzymic activity. The neuroglia are inactive. ×122.

FIG. 3.–*Toad.* Ventral horn. Acetylthiocholine substrate (90 min). The vascular endothelium of both grey and white matter, and the ventral motor neurons (N), all have marked AcThChE activity. Note the active lining of the ventromedian vessel (V). There is no appreciable neuroglial activity. ×60.

FIG. 4.—*Goldfish.* Dorsal and ventral horns of both sides. Butyrylthiocholine substrate (90 min). The vascular endothelium exhibits activity of both enzymes. In this section, BuThChE activity only is demonstrated. ×126.

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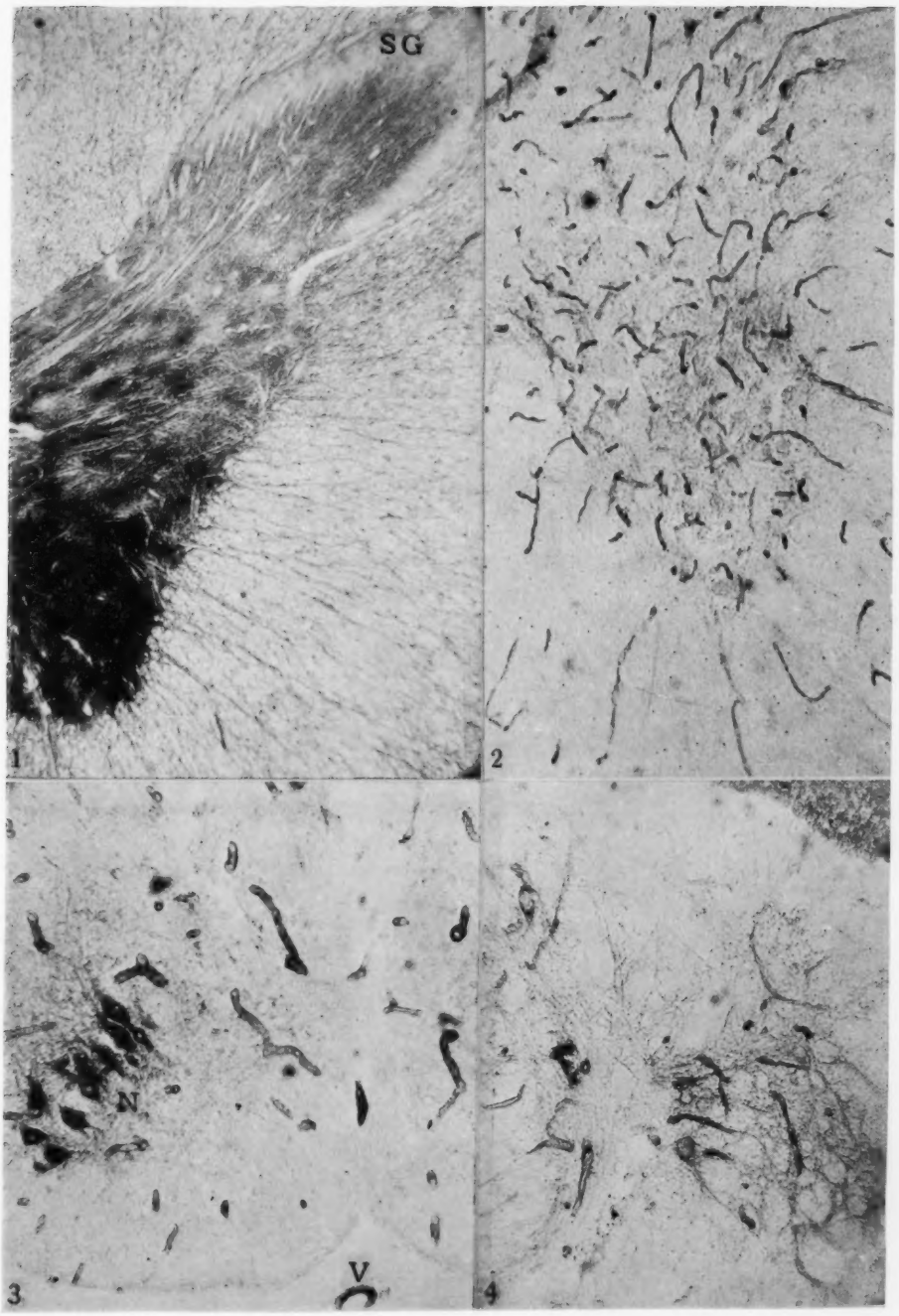

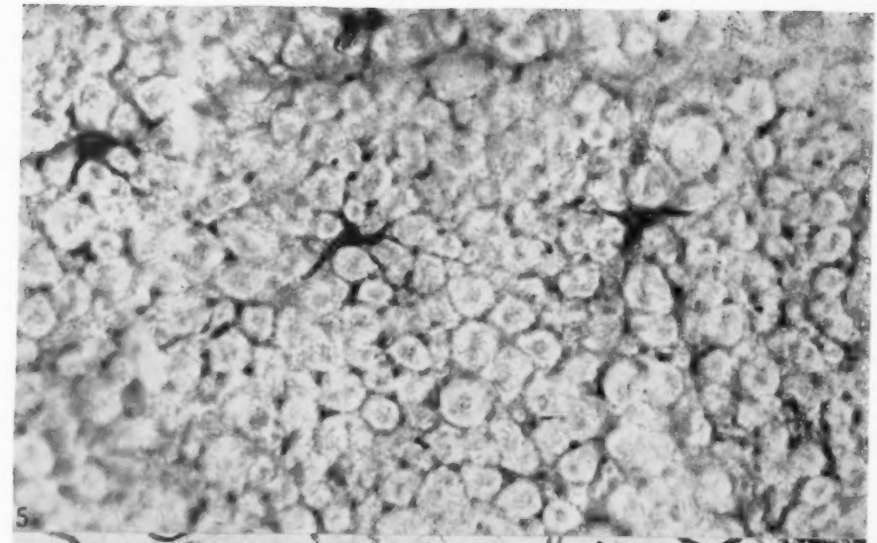

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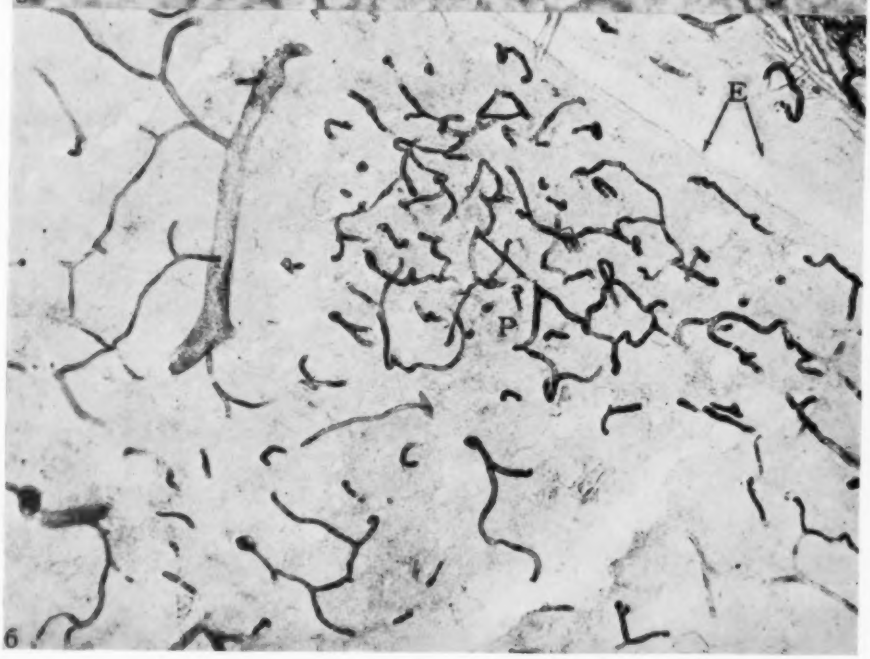

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of sodium pentobarbital. Several additional rats were similarly anaesthetized to determine whether or not the barbiturate affected the activity or distribution of the enzyme.

Within 5–15 min following anaesthesia or decapitation, pieces of cerebrum, hypothalamus, cerebellum, medulla oblongata and cervical, thoracic, and lumbar segments of the spinal cord were placed on strips of filter paper and immersed in liquid nitrogen. The tissue was stored in liquid nitrogen for about 16–24 hr, then mounted in a paste of homogenized brain tissue (LOWRY, 1953). Sections were cut at 22 μ with a Minot microtome housed in a cryostat which was maintained at $-15°$ to $-18°$.

The histochemical method of KOELLE (1951) was used, with a minor modification, throughout the experiment. The modification consisted in preparing the ammonium sulphide bath by adding 1 part of ammonium sulphide to 20 parts of distilled water, to which several drops of 0·1 M-copper glycine had been added, instead of saturating half-strength NH_4OH with H_2S, as in the original procedure.

The frozen, unfixed sections were removed from the cryostat, immediately plunged into a solution of 20% w/v Na_2SO_4, and then transferred directly into the appropriate storage solutions. The Na_2SO_4 and incubation solutions were adjusted to a pH of 6·0–6·4 by the addition of 0·2 M-K_2CO_3. Control sections were immersed in a storage solution of 24% w/v Na_2SO_4 containing 10^{-7} M-DFP, for about 30 min at 37·5° before incubation. Upon removal from the storage solutions, the sections were incubated at 38°, in either acetylthiocholine or butyrylthiocholine iodide solutions for 90 min in most cases, or for 60 min. Tissues from different species were mounted on the same or on consecutive cover slips and processed simultaneously in small staining jars. Throughout the experiment, additional sections of rat spinal cord were used as standards of the success of the experimental conditions, by incubating them with tissue from each of the other species.

Following immersion for 60 sec in the CuS solution, the sections were rinsed in distilled water and fixed for at least 10 min in 10% formalin. After rinsing in distilled water, the sections were dehydrated in ascending concentrations of ethanol, cleared in xylol, and mounted in 'permount' (Fisher).

OBSERVATIONS

After incubation with butyrylthiocholine, the vascular endothelium in control sections from the spinal cord of the rat always exhibited marked BuThChE activity. This activity ensured that absence of deposit in adjacent sections from other species was not due to the conditions of the experiment, but rather to an absence of appreciable enzymic activity. Barbiturate anaesthesia had no effect on the demonstration of either neuronal or extraneuronal cholinesterase.

The concentration of DFP in the acetylthiocholine solution was sufficient to inhibit BuThChE activity where present, but not the AcThChE activity. In all species examined, there was marked AcThChE activity in the neurons of brain and spinal cord. There was a striking species difference, however, in the distribution of extraneuronal cholinesterases (Table 1).

Sheep, monkey and man. BuThChE activity in all 3 species was slight as indicated by a faint, diffuse deposit of CuS. No elements recognizable as endothelial, neuroglia, or nerve cells were outlined.

Cat and rooster. The site of BuThChE in these two species is the neuroglia of the grey matter (Fig. 1) and white matter (Fig. 5). Because of their astral shape and

FIG. 5. *Cat.*—White matter. Butyrylthiocholine substrate (90 min). Three neuroglia cells, probably fibrous astrocytes, of the dorsal funiculi are outlined by CuS deposit. The processes of these cells surround the myelin sheath which, in turn, encloses the faintly discernible axon in the centre of each sheath. Cross-section. ×600.

FIG. 6.—*Rat.* Region of the paraventricular nucleus (P) of the hypothalamus; a portion of the third ventricle runs across the upper right corner of the field. Butyrylthiocholine substrate (90 min). The endothelium of the capillaries within this nucleus and of larger, adjacent vessels is highly active. The lumens of the vessels are relatively free of deposit. The ependyma (E) of the third ventricle and the neuroglia are inactive. ×149.

TABLE I— COMPARISON OF LOCI OF CHOLINESTERASE ACTIVITY IN VERTEBRATE CENTRAL NERVOUS SYSTEM

Site of Activity	Fish	Toad	Turtle	Rooster	Rat	Cat	Sheep	Monkey	Man
Neurons	AcThChE	AcThChE	AcThChE	AcThChE	AcThChE	AcThChE	—	AcThChE[2]	MChE[3]
Endothelium	AcThChE BuThChE	AcThChE	0	0	BuThChE	0	—	—	—
Neuroglia	0	0	0	BuThChE	0	BuThChE	BuThChE[1]	BuThChE[1]	BuChE[1,4]

A brown deposit of CuS denoted cholinesterase activity. The BuThChE of all species was sensitive to 10^{-7} M-DFP.
1 faint, diffuse activity not identifiable with any cell part.
0 absence of enzymic activity.
— material from this species was not incubated with AcThChE.
2 findings of KOELLE (1955).
3 hydrolysis of acetyl-β-methylcholine (MChE) by grey matter of basal ganglia (ORD and THOMPSON, 1952).
4 hydrolysis of butyrylcholine by subcortical white matter (ORD and THOMPSON, 1952).

location, the neuroglia cells of the funiculi were identified as astrocytes. The deposit of CuS in the neuroglia cells of the fowl was considerably less than in those of the cat.

Rat. The vascular endothelium of the brain (Fig. 6) and spinal cord (Fig. 2) is the site of BuThChE activity. Many anastomotic loops could be followed by virtue of this extensive activity; the lumens of the vessels were relatively free of granules even though the blood had not been washed from the vessels. The peri-endothelial tissue was entirely free of CuS granules.

The difference in location of BuThChE between cat and rat is further illustrated in Fig. 7, A, B, C: within the spinal cord, only the endothelium of the rat and the neuroglia of the cat are outlined by CuS deposit. The two species are similar, however, in that elements outside the central nervous system, e.g., within the spinal nerve roots, also exhibit BuThChE activity. The identification of these elements is uncertain, although their stellate shape and their location are suggestive of Schwann cells.

Turtle. Neither BuThChE nor extraneuronal AcThChE could be detected. In addition to the cell bodies of the ventral horn neurons, the nerve processes within the *substantia gelatinosa* exhibited intense AcThChE activity (cf. Fig. 1).

Toad. There was no appreciable BuThChE in either brain or spinal cord. Only a faint, diffuse precipitate of sulphide was formed in tissue incubated with butyrylthio-

FIG. 7.—Dorsal root of spinal nerve in cat and rat. Butyrylthiocholine substrate (120 min).
A. *Cat.* Phase contrast. A dorsal nerve root occupies the left half of the field. The dark, stellate elements in the root are presumably Schwann cells (S), the processes of which lie between the myelin sheaths of axons cut in cross section. There is a similar relation between neuroglia cell (G) and myelin sheath within the spinal cord, the periphery of which appears in the right half of the field. Between nerve root and spinal cord lies a blood vessel (B). The dark band in the upper left corner represents the arachnoid membrane which exhibits marked BuThChE activity. ×98.
B. Same area as in Fig. 7A. Bright field optics. The CuS granules are confined to the Schwann cells (S) of the nerve root and the neuroglia cells (G) within the spinal cord, where the deposit is only faintly discernible. The wall of the blood vessel (B) is practically invisible. ×98.
C. *Rat.* Bright field illumination. A dorsal root, the cut end of which appears in cross-section at either end of the field, lies adjacent to the periphery of the spinal cord, seen in the lower right corner. In the dorsal root cross-section the cell bodies of Schwann cells (S) are outlined by CuS, and their processes (P) are visible in the longitudinal section of nerve. In contrast, the neuroglia cells within the spinal cord are invisible. Only the endothelium of blood vessels such as the capillary (C) within the spinal cord are outlined by deposit. ×138.

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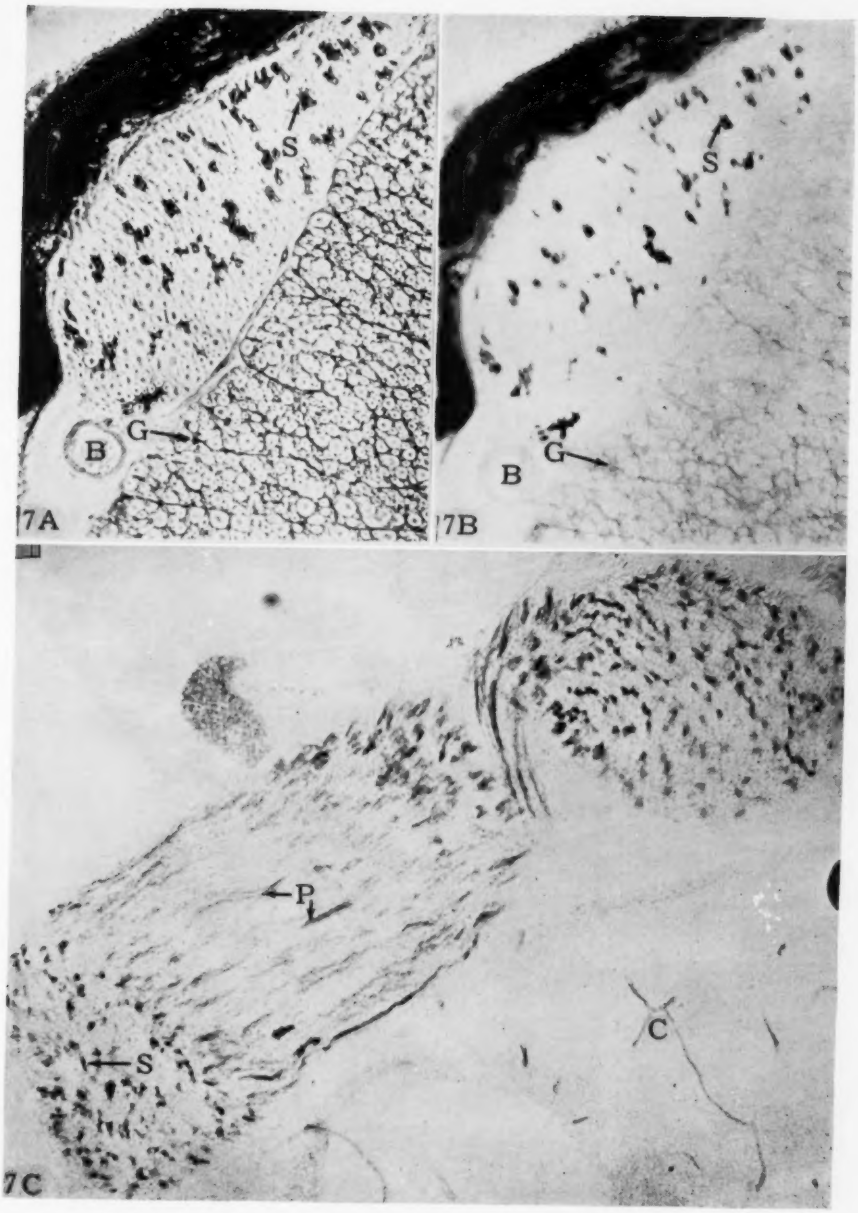

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choline. However, there was marked extraneuronal AcThChE activity within the vascular endothelium of brain and spinal cord. Intensely coloured neurons and blood vessels were demonstrated in the same section (Fig. 3).

Fish. The vascular endothelium exhibited *both* BuThChE and AcThChE activity in the spinal cord (Fig. 4), cerebrum and cerebellum. There was some diffuse colouring of the spinal cord tissue, but no neuroglia cells could be recognized.

DISCUSSION

The species differences in the extraneuronal distribution of AcThChE and of BuThChE do not follow an obvious phyletic sequence. A consideration of the enzymic activity of neuroglia and endothelium points to a number of similarities as well as to differences between species.

Endothelium. In some species, the locus of BuThChE is the blood vessel wall. Contribution to this activity by the plasma has been ruled out by previously washing the blood from the vessels (SHEN *et al.*, 1955). In the rat, the endothelium is the only extraneuronal element with BuThChE activity; none is detectable in the neuroglia (cf. KOELLE, 1952). This finding agrees with that of PEPLER and PEARSE (1957).

The isolated occurrence of AcThChE in the endothelium of the central nervous system is peculiar to the amphibian. The distribution in the toad is the same as that in the frog. In both anurans (SHEN *et al.*, 1955) and the urodele, i.e. *Amblystoma punctatum* (BOELL and SHEN, 1950), which were examined histochemically and manometrically, there are no esterases capable of hydrolysing either butyryl- or benzoylcholine. The possibility remains that unspecific esterases may be present which attack substrates other than those examined. The simultaneous occurrence of both AcThChE and BuThChE is a unique property of the endothelium in the brain and spinal cord of goldfish.

Neuroglia. The alternative site of BuThChE activity is the neuroglia cell. In the cat and rooster, this activity is confined to the neuroglia and does not occur in the endothelium (cf. KOELLE, 1950). In the rabbit, BuThChE is present also in the septae and interfascicular neuroglia of the optic nerve (HEBB *et al.*, 1953). In the dog brain, which was examined manometrically, BuThChE activity is associated with fiber tracts (BURGEN and CHIPMAN, 1951) and also with grey matter (ORD and THOMPSON, 1952).

Indirect evidence for the presence of a pseudocholinesterase in human neuroglia has been provided by manometric studies of normal material (ORD and THOMPSON, 1952) and of gliomas (BÜLBRING *et al.* 1953; CAVANAUGH *et al.*, 1954). CAVANAUGH *et al.* (1954) demonstrated only low activity in the cerebral vessels washed free of blood. Quantitative studies of plaques from the brains of patients with multiple sclerosis indicated no relationship between human oligodendrocytes or astrocytes and BuThChE activity (LUMSDEN, 1957). However, that such a relationship occurs in some species, is demonstrated by the present finding of this enzyme in the astrocytes of the cat and rooster. The negative histological findings in sheep, monkey (cf. KOELLE, 1955) and man may indicate only that in a given cell the enzymic activity is too slight to be detected histochemically under the limitations of the method. In addition to these limitations, a number of additional factors modify the histochemical detection of these enzymes.

Optimal pH. Except for the rat, in which the cholinesterases hydrolyse optimally at pH 6·2 (PEPLER and PEARSE, 1957), the Koelle method necessitates incubation at

a pH of about 1 unit below that required for optimal enzyme activity in a number of species.

Substrate specificity. Benzoylcholine is split by human cerebral white matter but not by comparable tissue from sheep or bovine species (BERNSOHN and POSSLEY, 1957). Similarly, four 'non-specific esterases' have been demonstrated in the rat brain in addition to the 'true' and 'pseudo' cholinesterases by the use of various substrates (PEPLER and PEARSE, 1957). In particular, it has been reported that the most rapidly hydrolysed substrate for rat brain preparations is proprionylcholine rather than butyrylcholine (ORD and THOMPSON, 1952).

Neither the natural substrate nor the function of extraneuronal cholinesterases are known. Several suggestions have been proposed, e.g. maintenance of the blood–brain barrier (GRIEG and HOLLAND, 1949; PAULET *et al.*, 1957) and maintenance of the myelin sheath (BARNES and DENZ, 1953). The second hypothesis is based on the results of the administration of certain organo-phosphorus compounds to some species. The ingestion of tri-*ortho*-cresyl phosphate by man or fowl is followed by demyelination of fibre tracts within the spinal cord (BARNES and DENZ, 1953). However, the apparent relation between prolonged inhibition of butyrylcholinesterase and loss of myelin is obscure, for a number of selective inhibitors do not bring about demyelination (DAVISON, 1953). Recent evidence points to a possible relation between lipid metabolism and this enzyme system. Acetylcholine stimulates the uptake of ^{32}P by the phospholipids of brain slices (HOKIN and HOKIN, 1955). Inhibition of BuThChE with one demyelinating agent, DFP, causes a retardation of lipid synthesis in the sciatic nerve of the chicken (AUSTIN, 1957). In contrast, the incorporation of inorganic ^{32}P into myelin lipids of the fowl does not appear to be affected by another demyelinating agent, tri-*ortho*-cresyl phosphate (WEBSTER, 1954). The possibility remains that extraneuronal cholinesterases may be involved indirectly in the normal metabolism of myelin.

A third suggested role of extraneuronal cholinesterases is one of neurohumoral 'scavenger' (KOELLE, 1950). Presumably, acetylcholine which has escaped splitting by AcThChE at the synapse and which has been released into the extraneuronal 'spaces', may be hydrolysed by AcThChE at the endothelium or by BuThChE at the vessel wall or neuroglia-cell membrane. This last site of action is plausible in view of the recent elucidation of the intimate anatomical association between neuroglia cells and neurons (PALAY, 1957; SCHEIBEL and SCHEIBEL, 1958).

The processes of perivascular astrocytes are closely applied to the endothelium at one pole and to the neuronal surface at another. This anatomical relationship has been taken to support the old concept of a transport of material to and from blood vessel, neuroglia and neuron (SCHEIBEL and SCHEIBEL, 1958). The passage of substances such as electrolytes across cell membranes is influenced by cholinesterase activity; e.g. the hydrolytic action of cholinesterase on the acetylcholine of guinea-pig heart muscle is accompanied by a loss of intracellular potassium. The inhibition of cholinesterase reduces the rate of this potassium loss (HOLLAND *et al.*, 1952). The presence of cholinesterase activity in the endothelium of some species and the neuroglia of other species further suggests a functional interrelationship between these cells. One might postulate that cholinesterase acts as a 'valve' in a transport mechanism which could function alternatively at the membrane of either one of these cells in the series: blood $\rightleftarrows$ endothelium $\rightleftarrows$ neuroglia $\rightleftarrows$ neuron. In this series, either

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endothelium or neuroglia cell could influence the transfer of substances to and from the neuron: although the site of the 'valve' is species dependent, the role in such a transport mechanism may be common to all species.

Recent physiological evidence also hints at a relationship between electrical and cholinesterase activities in the neuroglia cell. The electrical stimulation of cultured astrocytes from the cat brain results in a prolonged, electrical (TASAKI and CHANG, 1958) and contractile (CHANG and HILD, 1958) response on the part of these cells. *In vivo* stimulation with microelectrodes inserted into the cat's cerebral cortex has yielded similar responses, presumably from neuroglia cells (TASAKI and CHANG, 1958). It is conceivable that the satellite neuroglia cell, which itself is capable of electrical activity, may influence in some way the electrical activity of its neurons, and that BuThChE and other 'pseudo' cholinesterases may be implicated in this phenomenon. It would be informative to determine whether or not such activity is prevented by the use of selective inhibitors of butyrylcholinesterase.

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SUMMARY

The extraneuronal localization of BuThChE within the central nervous system of nine vertebrate species has been studied by the Koelle histochemical method. This enzyme occurs only in the fibrous astrocyte, in the spinal cords of cat and rooster, and only in the endothelium in the spinal cords of the rat and goldfish. There is no histologically demonstrable BuThChE activity in the spinal cords of amphibian and reptile and very little in the sheep, monkey and man.

AcThChE appears in the endothelium of some species (e.g. toad and goldfish), but not in the neuroglia of any of the species examined. The simultaneous occurrence of both BuThChE and AcThChE in the endothelium was found only in the goldfish. These striking species differences in the localization of extraneuronal cholinesterases do not follow an obvious phyletic pattern.

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THE EFFECT OF RESERPINE ON THE INTRACELLULAR DISTRIBUTION OF CATECHOLAMINES IN THE BRAIN STEM OF THE RABBIT

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THE discovery of the action of reserpine on cerebral serotonin and catecholamines promises to be of great value for a better understanding of the function of these neuro-hormones. According to current concepts reserpine releases the amines from a bound form, thus exposing them to rapid destruction by amine oxidase; if amine oxidase is inhibited the depleting effect of reserpine is reduced and its tranquilizing effect is suppressed or changed into an excitatory one, presumably owing to the intracellular accumulation of amines in their free form (BRODIE, PLETSCHER and SHORE, 1956; BRODIE, OLIN, KUNTZMAN and SHORE, 1957; PLETSCHER, SHORE and BRODIE, 1956; SHORE, PLETSCHER and BRODIE, 1956).

Nothing is known about the process which transforms the bound into the free form, but an obvious assumption is that it involves a transfer of amines from particles, such as mitochondria, into the surrounding cellular plasma. Such an assumption is supported by several facts: (1) it is known that the catecholamines in the adrenal medulla are largely contained in particles from which they are discharged into the blood stream (HILLARP and NILSON, 1954; BLASCHKO, HAGEN and WELCH, 1955). In other tissues too, and particularly in the brain (WEIL-MALHERBE and BONE, 1957*b*), a considerable proportion of catecholamines is located in particles. (2) Reserpine causes the release of serotonin from blood platelets, *in vivo* (SHORE *et al.*, 1956) and *in vitro* (CARLSSON, SHORE and BRODIE, 1957); it also blocks the uptake of serotonin by platelets *in vitro* (HUGHES, SHORE and BRODIE, 1958). (3) Acetylcholine, like serotonin and the catecholamines, is assumed to exist mainly in a bound form in tissues, and there is evidence to show that the bound form is 'sequestered' within particles associated with the mitochondrial fraction (STONE, 1955; HEBB and WHITTAKER, 1958; WHITTAKER, 1958).

The effect of reserpine on the release and the intracellular redistribution of catecholamines in the brain stem of the rabbit was therefore investigated. It has previously been shown that brain, in addition to adrenaline and noradrenaline, contains hydroxytyramine which accounts for about 50 per cent of the total catecholamines present (MONTAGU, 1957; WEIL-MALHERBE and BONE, 1957*b*; CARLSSON *et al.*, 1958). Each of the three catecholamines in cerebral tissue was shown to be about evenly distributed between a particulate and a soluble fraction (WEIL-MALHERBE and BONE, 1957*b*).

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The effect of reserpine on the intracellular distribution of catecholamines could obviously be studied only if the depletion was incomplete so that the residue could be measured with accuracy. The dose of reserpine was, therefore, chosen so as to produce a disappearance of about 50 per cent of catecholamines in 4 hr. A preliminary account of the principal findings has been published (WEIL-MALHERBE and BONE, 1958*b*).

METHODS

Fractionation of tissue

Rabbits weighing 2–3 kg were used in the experiments. The brain was removed and medulla oblongata, cerebellum and cerebral cortex with the underlying white matter down to the lateral ventricles were discarded. The remaining parts, consisting of pons, midbrain, thalamus, hypothalamus and corpus striatum, were weighed and homogenized in 9 vols. of a solution of 10% (w/v) sucrose and 0·1% (w/v) sodium ethylenediaminetetra-acetate (pH 7·0). One-quarter of the total volume was acidified immediately by the addition of 0·1 vol. of 1·0 N-HCl and set aside for the analysis of the whole homogenate. The remaining three-quarters were separated into three fractions as follows: fraction 1, containing whole cells, nuclei and debris, was obtained by centrifuging at 0° in the angle head 6886 of the MSE refrigerator centrifuge (Measuring and Scientific Equipment Ltd., London, England) at 1500 rev/min (300 **g**) for 5 min. Fraction 2, containing mainly mitochondria, was obtained by centrifuging the supernatant from fraction 1 at 13,000 rev/min (10,000 **g**) for 20 min (MSE Superspeed Unit). Fraction 3, containing microsomes and cytoplasm, was the supernatant from fraction 2. Since the microsomes were previously shown to contain little, if any, catecholamines (WEIL-MALHERBE and BONE, 1957*b*), their separation from the cytoplasmic fraction was deemed unnecessary. The sediments from fractions 1 and 2 were resuspended in a volume of 0·1 N-HCl equal to that before centrifugation; fraction 3 was acidified with 0·1 vol. of 1·0 N-HCl.

Separation of acid and basic catechol fractions

After standing overnight at 0° fractions 1 and 2, as well as the sample of whole homogenate, were centrifuged. To the supernatant solutions and also to fraction 3 were added 0·1 vol. of 1% (w/v) disodium ethylenediaminetetra-acetate and 0·1 vol. of a freshly prepared 1% (w/v) solution of ascorbic acid. A micro glass electrode assembly was inserted and the solutions, while being vigorously stirred magnetically, were titrated to pH 5 with 1·0 N-NaOH and then to pH 8·4 with 0·5 N-Na_2CO_3. Precipitates which formed upon neutralization were centrifuged off and the supernatants passed over columns of alumina. The preparation of the columns and the technique of adsorption, washing and elution were the same as described in previous reports (WEIL-MALHERBE and BONE, 1952, 1957*a*, 1958*a*).

The eluates from the alumina columns, containing catechols with basic, acid and, possibly, neutral side chains, were mixed with 0·1 vol. of 1% (w/v) disodium ethylenediaminetetra-acetate to prevent the precipitation of traces of aluminium hydroxide; they were then brought to pH 6·5 and passed over columns of 0·5 g of damp Amberlite IRC-50, buffered at pH 6·5 (BERGSTRÖM and HANSSON, 1951). This weakly acidic cation exchange resin retained only the catecholamines, while acid or amphoteric catechol compounds passed into the filtrate. The columns were washed with 40 ml water and eluted with 10 ml of 1·0 N-HCl. The filtrates and washings were combined and set aside for further analysis. The three catecholamines were contained in the eluates.

Elution of the alumina columns by the standard procedure (5 ml of 0·2 N-acetic acid followed by 5 ml of water), though adequate for the recovery of the basic fraction, gave somewhat low recoveries for 3:4-dihydroxyphenylalanine (DOPA) and 3:4-dihydroxyphenylacetic acid (DOPAC). Recoveries were improved when the elution of the alumina columns was continued with 30 ml of 0·2 N-H_2SO_4. This portion was collected separately and pooled with the filtrate and washings from the Amberlite columns.

DOPA was separated from other acid catechols on columns of 0·5 g of damp Zeo-Karb 225, a strongly acidic cation exchange resin which was used in the H^+-form. Before the solutions were put on the columns they were briefly evacuated in order to prevent the appearance of CO_2-bubbles during the passage through the columns. The columns were washed with 50 ml water and eluted with 25 ml 0·2 N-$BaCl_2$ (WEIL-MALHERBE and BONE, 1957*a*). The eluates contained DOPA while

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other acid catechols were present in the filtrates. The eluates were freed from barium by adding 1 ml of 10 N-H_2SO_4. Filtrates and eluates were adjusted to pH 3 and concentrated to 10 ml *in vacuo*.

Fig. 1 shows a diagram of the fractionation procedure. A complete analysis in which water was substituted for tissue extract was performed with each run and the results were corrected accordingly.

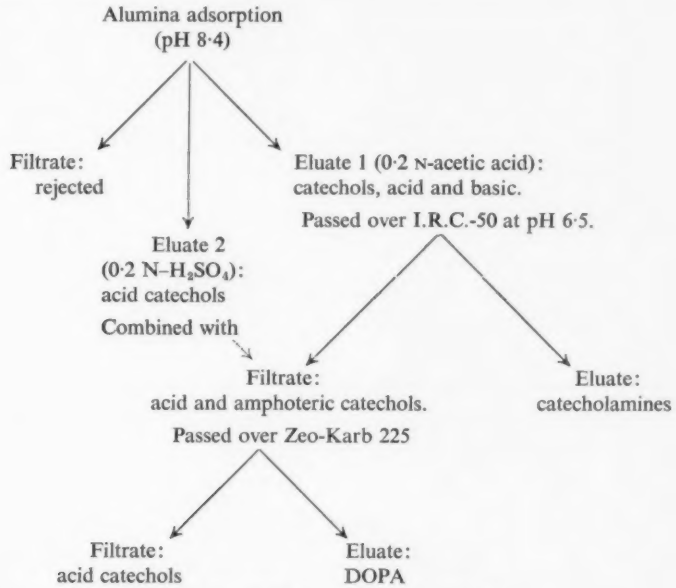

FIG. 1.—Scheme for chemical fractionation of tissue extracts.

Fluorimetric analysis

(a) *Basic fraction.* The three catecholamines were estimated without prior separation by a combination of two fluorimetric methods: adrenaline and noradrenaline were determined by the trihydroxyindole method; the ethylenediamine condensation method was performed on another aliquot of the solution, yielding fluorescent derivatives of adrenaline, noradrenaline and hydroxytyramine. Hydroxytyramine was measured by subtracting the contribution of adrenaline and noradrenaline from the total fluorescence. For a discussion of the principle and the specificity of the method and for details of the procedure see WEIL-MALHERBE and BONE (1957*a*).

The eluates containing the amine fraction were adjusted to pH 3·5 with 1·0 N-NaOH and diluted to 25 ml. Samples of 2–5 ml were used for the fluorimetric tests. In earlier experiments adrenaline and noradrenaline were differentiated by oxidation at pH 3·5 and 6·0, respectively, according to EULER and FLODING (1955); later a method of optical differentiation was used, after oxidation at pH 6·0 (COHEN and GOLDENBERG, 1957). The two filter combinations used were as follows: (1) Wratten no. 35 + no. 44 as primary and Ilford Bright Spectrum 623 as secondary filters, and (2) Chance OB 10 as primary and Chance OY 4 as secondary filters. The second set of filters was also used for the measurement of the fluorescence of the ethylenediamine condensation products. Recoveries of adrenaline, noradrenaline and hydroxytyramine after addition to homogenates were 80–90 per cent.

(b) *DOPA.* DOPA was determined by condensing the eluate from the Zeo–Karb column with ethylenediamine, using the same conditions as for the estimation of adrenaline. The mixture was extracted with *iso*butanol, but only the aqueous phase, which contains about 90 per cent of the fluorescent derivative of DOPA, was used for fluorimetry. Interference from contamination with catecholamines was thereby minimized. Recoveries of DOPA were 70–80 per cent.

(c) *Acid catechols.* These were estimated in the filtrate from the Zeo–Karb column by condensation with ethylenediamine. Usually this fraction was too small to be measured with confidence and its composition was therefore not investigated in detail. Significant readings were only obtained after the administration of DOPA and were presumably mainly due to the formation of DOPAC.

The estimations were based on the fluorescence of the aqueous phase, after extraction with *iso*butanol and the results were expressed in terms of DOPAC although it should not be implied that DOPAC was the only compound measured.

MATERIALS

Amberlite IRC-50, analytical grade, 100–200 mesh, was obtained from British Drug Houses Ltd.; Zeo-Karb 225, 100–200 mesh, water regain 1·8 (corresponding to 4·5 per cent cross-linking) from the Permutit Company, London W.4. Samples of adrenaline, noradrenaline, 3-hydroxytyramine and L-DOPA were obtained from commercial sources. DOPAC was prepared by an adaptation of the method of ABBOTT and SMITH (1949). Iproniazid (Marsilid; 1-*iso*nicotinyl-2-*iso*propylhydrazide) was a gift from Roche Products Ltd., Welwyn Garden City, England. The reserpine used was 'Serpasil'-Ciba.

RESULTS

Inhibition of amine oxidase

The concentration of catecholamines and particularly of hydroxytyramine is considerably smaller in a sample of brain that has been homogenized and fractionated in sucrose before acidification than in a sample homogenized in acid. This is presumably due to the action of amine oxidase in the interval between death and acidification. The loss can be almost completely prevented by the injection of iproniazid (100 mg/kg intraperitoneally) 1 hr before death (Table 1). No further protection was obtained

TABLE 1.—EFFECT OF THE INJECTION OF IPRONIAZID ON THE CONCENTRATION AND STABILITY OF ADRENALINE (A), NORADRENALINE (N) AND HYDROXYTYRAMINE (H) IN RABBIT BRAIN

Expt. no.	Iproniazid injected 1 hr before death (100 mg/kg, i.p.)	Cerebral tissue homogenized in 0·1-N HCl			Cerebral tissue homogenized in 10% sucrose, left at 0° for 1 hr, then acidified		
		A	N	H	A	N	H
		(μg/kg)					
(1)	–	51	152	278	21	56	62
(2)	–	71	184	214	41	111	59
(3)	+	89	320	776	77	278	750

by adding iproniazid *in vitro* before homogenizing (Table 2). Iproniazid was therefore injected in all experiments, either 1 hr or 6 hr before death (see Table 3).

Effects of reserpine

Two series of experiments were carried out. In both of them reserpine was injected intravenously 4 hr before death; iproniazid, on the other hand, was injected either 1 hr or 6 hr before death. In the first series of experiments, therefore, the action of reserpine was not modified by the inhibition of amine oxidase for at least 3 hr, while in the second series amine oxidase was inhibited throughout the action of reserpine.

The dose of reserpine required to produce a 50 per cent depletion of catecholamines was different in the two series. When iproniazid was injected after reserpine, 0·25–0·5 mg/kg were found adequate; this dose was doubled when the sequence was

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TABLE 2.—THE EFFECT OF ADDING IPRONIAZID *in vitro* BEFORE FRACTIONATION. IPRONIAZID (100 mg/kg, I.P.) WAS INJECTED 6 HR BEFORE DEATH

Fraction	Cerebral tissue homogenized in 10% sucrose			Cerebral tissue homogenized in solution containing 10% sucrose +0·001 M-iproniazid		
	A	N	H	A	N	H
	(μg/kg)					
Whole homogenate	48	193	284	42	196	248
Coarse	10	19	62	7	41	54
Particulate	16	64	107	13	60	87
Supernatant	13	61	89	15	52	97

A = adrenaline, N = noradrenaline, H = hydroxytyramine.

TABLE 3.—THE INTRACELLULAR DISTRIBUTION OF CATECHOLAMINES IN THE BRAIN STEM OF THE RABBIT AND THE EFFECTS OF VARIOUS DRUGS ON IT

Experimental group	Treatments: Amounts of drugs injected/kg and time of injection before death			No. of experiments	Fraction	Adrenaline	Noradrenaline	Hydroxytyramine
	Reserpine (intravenous)	Iproniazid (intraperitoneal)	Dihydroxyphenylalanine (intravenous)			(μg/kg wet weight)		
(1)	0	100 mg, 1 hr	0	8	Whole	82·3 ± 9·2	311·5 ± 38·4	417·6 ± 90·0
					Coarse	11·1 ± 1·0	47·2 ± 7·3	59·9 ± 11.8
					Particulate	31·0 ± 4·0	119·3 ± 16·5	148·1 ± 36·9
					Supernatant	28·7 ± 3·3	106·9 ± 12·2	157·0 ± 52·0
(2)	0·25–0·5 mg, 4 hr	100 mg, 1 hr	0	10	Whole	38·2 ± 8·3	223·3 ± 49·6	141·7 ± 48·3
					Coarse	5·5 ± 1·6	28·8 ± 7·2	24·9 ± 7·3
					Particulate	18·3 ± 3·5	114·5 ± 25·6	68·8 ± 20·5
					Supernatant	10·8 ± 2·9	57·2 ± 9·9	60·8 ± 19·2
(3)	0·5–1·0 mg, 4 hr	100 mg, 6 hr	0	8	Whole	31·7 ± 6·1	157 ± 15·5	173 ± 58·3
					Coarse	5·7 ± 1·2	30·4 ± 4·9	33·4 ± 13·8
					Particulate	14·9 ± 3·1	66·0 ± 7·9	87·9 ± 28·3
					Supernatant	8·2 ± 1·6	41·3 ± 5·3	41·0 ± 15·0
(4)	0	100 mg, 2 hr	60 mg 1 hr	2*	Whole	130, 100	303, 268	4590, 13420
					Coarse	10, 12·6	61, 45·5	643, 1520
					Particulate	44, 32·4	109, 87·5	1400, 4540
					Supernatant	52, 35·7	102, 96·8	2110, 6160
(5)		100 mg, 6 hr	60 mg, 5 hr	2	Whole	79, 111	215, 490	1740, 1270
					Coarse	12·3, 16·5	35·4, 85	208, 207
					Particulate	38·6, 24·6	80·6, 191	768, 414
					Supernatant	33·8, 31·6	83, 147	345, 273
(6)	0·5 mg, 4 hr	100 mg, 6 hr	60 mg, 0·5–1 hr	3	Whole	90·8 ± 15·7	239 ± 19·5	4350 ± 1187
					Coarse	17·4 ± 4·8	40·8 ± 4·7	779 ± 297
					Particulate	33·1 ± 5·1	78·8 ± 7·8	1761 ± 645
					Supernatant	39·2 ± 3·9	105·2 ± 13·2	2370 ± 602
(7)	0·5 mg, 4 hr	100 mg, 6 hr	60 mg, 5 hr	4	Whole	68·9 ± 8·5	293 ± 12·1	2400 ± 665
					Coarse	12·0 ± 1·3	56 ± 12·2	350 ± 107
					Particulate	34·8 ± 5·3	132 ± 6·6	960 ± 300
					Supernatant	22·2 ± 5·5	86 ± 7·8	975 ± 296

(The figures are means ± S.E.M., except in groups (4) and (5) where both results are shown)
* These animals died spontaneously 1 hr after the injection of dihydroxyphenylalanine.

reversed (Table 3). The correct dose level was established by trial and error. Satisfactory results were obtained within the range of doses stated. The results of different experiments within these ranges were pooled although this probably increased their scatter.

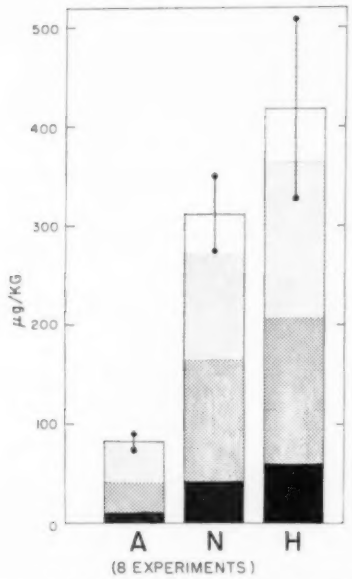

FIG. 2.—Concentration of catecholamines in fractions of rabbit brain stem (control series).

The total height of the columns indicates the concentrations in the whole homogenate. Coarse fraction shown by black, particulate fraction by cross-hatched and supernatant fraction by stippled parts of column. The open area shows the amount unaccounted for after fractionation.

Vertical bars indicate standard errors of the mean.

A = adrenaline, N = noradrenaline, H = hydroxytyramine.

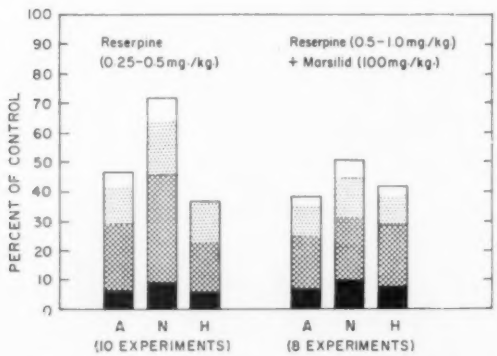

FIG. 3.—Concentrations of catecholamines in fractions of rabbit brain stem, expressed in percentages of the control values, after treatment with reserpine.

On left experimental group No. 2 (iproniazid 1 hr. before death), on right group No. 3 (iproniazid 6 hrs. before death) (cf. Table 3).

Notation of subcellular fractions as in Fig. 2.

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The results are shown in Table 3 and in Figs. 2 and 3. Fig. 2 shows the concentrations and the intracellular distribution of catecholamines in the brain of untreated controls in μg/kg, whereas Figs. 3–5 were constructed as follows: the results obtained with the total homogenate were expressed as percentages of the control values and the results found for the subcellular fractions were expressed in proportion to the concentrations in the total homogenate.

On the basis of the assumption that reserpine releases catecholamines from a particle-bound state, one would have expected the particulate fraction to disappear at a faster rate than the cytoplasmic fraction. Inhibition of amine oxidase by an injection of iproniazid preceding that of reserpine should have accentuated this difference since the releasing effect of reserpine would presumably have continued without the concomitant destruction of the 'free' fraction. However, apart from the fact that iproniazid may have increased the reserpine requirement, the results in the two series of experiments were not much different. Contrary to expectation, the rate of disappearance was greater in the cytoplasmic than in the particulate fraction, whether amine oxidase was inhibited or not. In Fig. 6 the concentrations in the cytoplasmic fraction are shown as percentages of those in the particulate fraction. In the control experiments the values are close to 100, while in the reserpine-treated animals they are significantly less than 100, with one exception owing to two outlying results which greatly increased the scatter. It is noteworthy that the depleting effect of reserpine is similar for all three amines (Fig. 3).

The effect of intravenous DOPA, with and without reserpine

It has been suggested (BRODIE *et al.*, 1956) that reserpine, supplementary to its releasing action, blocks the entry of newly synthesized amines into the particles. The effect of intravenous L-DOPA on the intracellular distribution of catecholamines in the brain of normal and reserpinized animals was therefore investigated.

It is known that the intravenous injection of L-5-hydroxytryptophan leads to an increase of cerebral serotonin while that of serotonin does not, presumably because 5-hydroxytryptophan but not serotonin is able to cross the blood–brain barrier (UDENFRIEND, WEISSBACH and BOGDANSKI, 1957; FRETER, WEISSBACH, UDENFRIEND and WITKOP, 1957). The experiments of HOLTZ, BALZER and WESTERMANN (1957) and CARLSSON, LINDQVIST and MAGNUSSON (1957), demonstrating central effects of DOPA, suggested a similar situation in the case of DOPA and the catecholamines.

L-DOPA, in 0·66% (w/v) aqueous solution, was injected through an ear vein in doses of 55–60 mg/kg, either 0·5–1 hr or 5 hr before death; in addition, all animals received an injection of iproniazid, usually given 6 hr before death. It was found necessary, in order to avoid excessive excitation and to facilitate the injection, to anaesthetize the rabbits; for this purpose they received sodium pentobarbital 50 mg/kg, intraperitoneally, 15 min before the injection of DOPA. After the injection of DOPA the animals remained unconscious for some time and after wakening lay quietly in their cages. Breathing was rapid and shallow, sometimes laboured and noisy.

In four experiments DOPA was injected 1 hr after iproniazid. Two of these animals died 1 hr later. Nevertheless, the brains were analysed and the results are included in Table 3 and in Figs. 4 and 5. The other two animals were killed 5 hr after the injection of DOPA. The effect of DOPA on reserpine-treated animals was

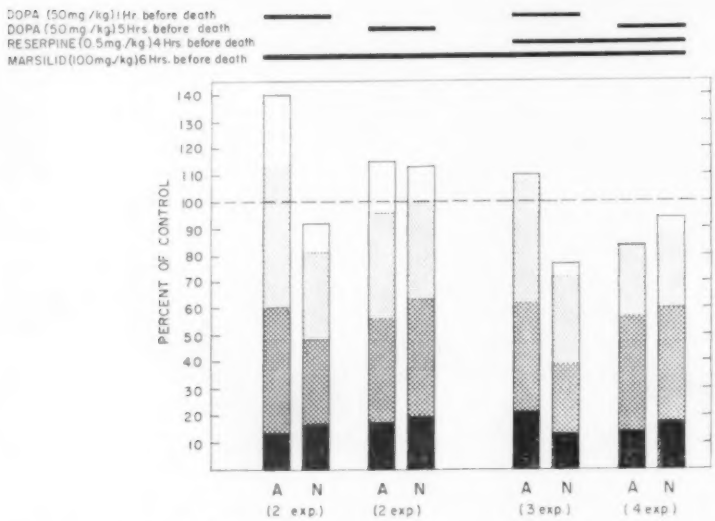

FIG. 4.—The effect of DOPA on the concentrations of adrenaline (A) and noradrenaline (N) in rabbit brain.

On left experimental groups No. 4 and 5, on right groups No. 6 and 7 (cf. Table 3). The height of the columns indicates mean values expressed as percentages of the mean control values. Notation of subcellular fractions as in Fig. 2.

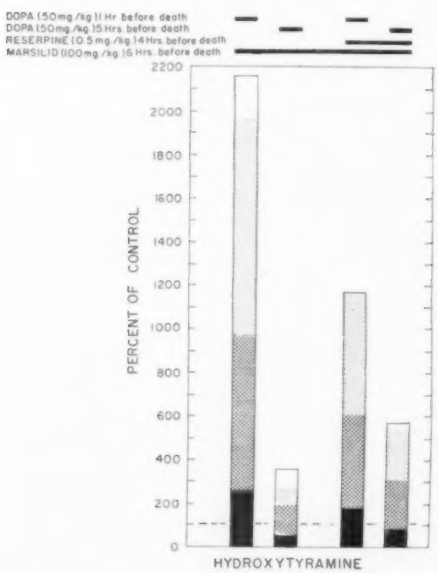

FIG. 5.—The effect of DOPA on the concentration of hydroxytyramine in rabbit brain. For explanation see legend of Fig. 4.

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investigated in another series of experiments: iproniazid was injected 6 hr and reserpine 4 hr before death; the time interval between the injection of DOPA and death was 5 hr in four animals and 0·5–1 hr in three animals. No spontaneous deaths occurred among the reserpine-treated animals; in fact, reserpine seemed to alleviate the effects of DOPA.

In animals not treated with reserpine the concentrations of adrenaline and noradrenaline were either unchanged or slightly increased after the injection of DOPA. The concentration of hydroxytyramine, on the other hand, was greatly increased, especially 1 hr after the injection when it was about twenty times normal. After 5 hr it had subsided to about three times normal. The distribution ratio of hydroxytyramine between particles and supernatant was about 2 : 3 1 hr, and about 3 : 2 5 hr after the injection of DOPA. The distribution ratios of adrenaline and noradrenaline did not deviate considerably from those of the controls.

In order to evaluate the effect of DOPA on reserpinized animals the results should be compared with those of the experimental series no. 3. It appears that the concentrations of adrenaline, noradrenaline and hydroxytyramine, determined in the whole homogenate, are significantly increased, 1 hr as well as 5 hr after the injection of DOPA ($P < 0{\cdot}01$ in every case). Moreover, there is a significant increase of all three amines in the particulate fraction 5 hr ($P = 0{\cdot}02$ to $< 0{\cdot}01$), and also, with the exception of noradrenaline, 1 hr after the injection of DOPA. Reserpine, therefore, did not block the uptake of newly-formed amines by the particles.

If the results obtained after injecting DOPA into reserpine-treated animals are compared with those of the normal controls it appears that DOPA has raised the concentrations of adrenaline and noradrenaline to about the normal level, in the total homogenate as well as in the subcellular fractions; that of hydroxytyramine is elevated far above the normal level.

As for the intracellular distribution of the catecholamines, the relative depletion of the cytoplasmic fractions caused by reserpine has disappeared 1 hr after the injection of DOPA; 5 hr after the injection of DOPA the distribution of adrenaline and noradrenaline is again similar to that in animals treated with reserpine only (Fig. 6).

DOPA and DOPAC in brain fractions

We have been unable to detect significant amounts of DOPA or DOPAC in any experiment except after the injection of DOPA. Even then the results were variable and a satisfactory agreement between the concentration in the whole homogenate and the sum of the concentrations in the subcellular fractions could not always be obtained. We have therefore selected four results, one from each of the experimental groups 4–7, which we regard as reliable. They are shown in Table 4 and they indicate a tendency for DOPA to accumulate in the particulate fraction while DOPAC is mainly located in the cytoplasm.

DISCUSSION

The depleting action of reserpine on brain noradrenaline was first described by HOLZBAUER and VOGT (1956). According to the present observations, reserpine also acts on adrenaline and hydroxytyramine in brain; moreover, the three catecholamines were shown to disappear at a similar rate.

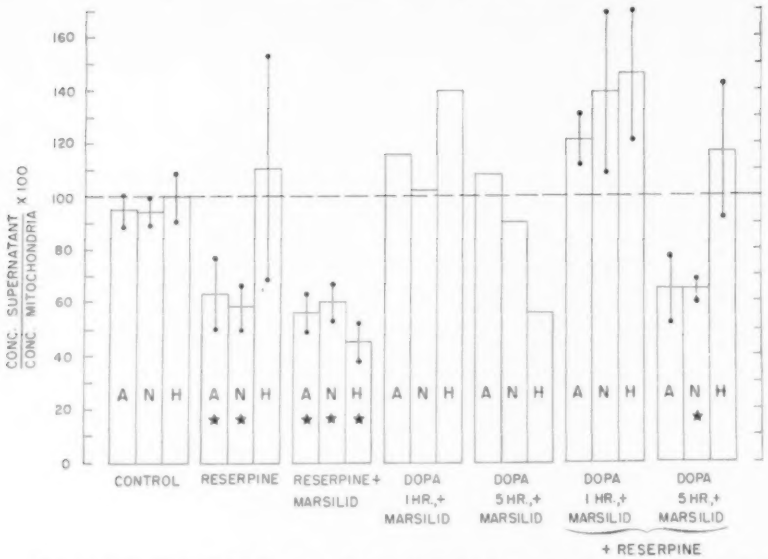

FIG. 6.—Supernatant fractions of brain catecholamines, expressed as percentages of the particulate fractions, in experimental groups 1–7.
A = adrenaline, N = noradrenaline, H = hydroxytyramine.
Vertical bars indicate standard errors of the mean. Columns with asterisks are significantly lower than 100 ($P < 0{\cdot}01$).

TABLE 4.—CONCENTRATIONS OF 3:4-DIHYDROXYPHENYLALANINE (DOPA) AND 3:4-DIHYDROXYPHENYLACETIC ACID (DOPAC) IN THE BRAIN STEM OF RABBITS AFTER INJECTION OF DOPA AND IPRONIAZID
(See Table 3 for experimental details)

Experimental group no.	Fraction	DOPA	DOPAC
		(μg/kg)	
(4)	Whole	4600	1930
	Coarse	650	110
	Particulate	1170	320
	Supernatant	2440	1490
(5)	Whole	1940	4100
	Coarse	170	430
	Particulate	1620	440
	Supernatant	350	3550
(6)	Whole	15,000	1800
	Coarse	1160	426
	Particulate	9500	480
	Supernatant	5700	1070
(7)	Whole	3280	1020
	Coarse	210	340
	Particulate	2350	240
	Supernatant	0	930

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The study of the intracellular distribution of the catecholamines in brain showed that in control animals they are distributed evenly between particles and cytoplasm. Since the volume of the particles is smaller than that of the cytoplasm, the actual concentration of catecholamines is higher in the particles. The concentration gradient is probably maintained by an active process of catecholamine uptake. It has been suggested (HUGHES, SHORE and BRODIE, 1958) that reserpine acts by paralysing this active transport mechanism. As a result, amines would diffuse out of the particles and, especially when amine oxidase is inhibited, the concentration in the cytoplasmic fraction would rise.

It is obvious that this hypothesis is not in accord with the facts as here observed, since the cytoplasmic fraction was found to disappear more rapidly than the particle-bound fraction; this was the case even when amine oxidase was inhibited. There is, therefore, no reason to assume that the effect of reserpine is preceded by, and dependent on, an intracellular redistribution of catecholamines. Our data do not indicate where the accelerated breakdown takes place: it may be in the particles, in the cytoplasm or in both. If it is restricted to one of the two fractions, any resulting concentration differences may be obliterated by a secondary redistribution of amines.

Unless reserpine acts by releasing the amines from some form of restriction other than their association with particles (e.g. a chemical complex), the facts can be explained by assuming that reserpine directly activates amine oxidase. This it could do by releasing the enzyme from some physical or chemical bond or inhibition. Such a mechanism would be in accordance with the effect of iproniazid which acted as a simple inhibitor of reserpine in our experiments and also in the histochemical studies of ZBINDEN and STUDER (1958).

The effects of reserpine on brain catecholamines are counteracted not only by iproniazid, but also by DOPA. While the former inhibits breakdown, the latter enhances synthesis. The spontaneous restoration of noradrenaline concentration in the brain, after maximal depletion, takes a week or more (BRODIE *et al.*, 1957). Spontaneous restoration after 50 per cent depletion would presumably have taken several days. Yet a significant restoration of adrenaline and noradrenaline concentration was achieved within an hour of the injection of DOPA, suggesting that brain tissue is capable of a rapid synthesis of both adrenaline and noradrenaline and that, after depletion by reserpine, the rate of synthesis is limited by the supply of DOPA.

It has not been possible to raise the concentration of adrenaline and noradrenaline much above the normal level by the administration of DOPA and iproniazid, either in untreated or in reserpine-treated animals; that of hydroxytyramine, on the other hand, was dramatically increased in both. Even 5 hours after the injection of DOPA the concentration of hydroxytyramine was 3–5 times as high as the normal level. In spite of this, the concentrations of adrenaline and noradrenaline did not rise further between the first and fifth hour after DOPA administration. This seems to indicate that, having reached the normal level within the first hour, the concentrations of adrenaline and noradrenaline were close to the saturation level of the tissue. The capacity of brain tissue for hydroxytyramine, on the contrary, is far above its normal concentration level.

There is no doubt that the administration of DOPA produced a significant uptake of catecholamines into the particles and that this process was not prevented by

reserpine at the doses used in our experiments. It is possible that higher doses would have had this effect; the problem will be further investigated.

It is interesting, finally, to notice the difference in the intracellular distribution of DOPA and DOPAC: while DOPA tends to accumulate in the particulate fraction, DOPAC is mainly localized in the cytoplasm. DOPAC is presumably an end product of metabolism and without value from the point of view of cellular economy. This distribution pattern is therefore in agreement with the view that one of the functions of the particulate fraction is that of a storage depot.

SUMMARY

The intracellular distribution of adrenaline, noradrenaline and hydroxytyramine in the brain stem of the rabbit and the effect of reserpine thereon has been examined. In untreated animals the three catecholamines are present in the ratio of 1 : 4 : 5, and each of them is about evenly distributed between a particulate fraction, consisting mainly of mitochondria and the cytoplasm.

Reserpine was used in doses which caused the disappearance of approximately 50 per cent of catecholamines within 4 hours; the rate of disappearance was similar for all three amines. The cytoplasmic fraction was found to be depleted more rapidly than the particulate fraction, whether amine oxidase had been inhibited by a prior injection of iproniazid or not. Iproniazid may have increased the required dosage of reserpine but did not affect the change in the intracellular distribution of catecholamines produced by reserpine. These results therefore do not support the hypothesis that reserpine acts by releasing amines from a particle-bound form.

The intravenous injection of L-3:4-dihydroxyphenylalanine (DOPA), in reserpinized animals, restored the concentration of adrenaline and noradrenaline to about the normal level within an hour. The concentration of hydroxytyramine was increased far above the control level. The concentrations of all catecholamines were increased in both cellular fractions, showing that reserpine did not prevent the uptake of amines by the particles. Five hours after the injection of DOPA the concentration of hydroxytyramine was still five times as high as in the controls although there was no further increase of adrenaline or noradrenaline. The effect of DOPA on brain catecholamines in untreated animals was similar: it caused a dramatic increase of hydroxytyramine, but adrenaline and noradrenaline were not significantly raised above their normal level. It was concluded that brain tissue is capable of rapid synthesis of adrenaline and noradrenaline if DOPA is supplied, but that the normal concentrations are close to the saturation level of the tissue.

Neither DOPA nor 3:4-dihydroxyphenylacetic acid (DOPAC) were found in brain tissue except, sometimes, after the injection of DOPA. DOPA was found to accumulate in the particulate fraction while DOPAC was mainly located in the cytoplasm.

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EFFECT OF DENERVATION ON HISTOCHEMICALLY DEMONSTRABLE ACETYLCHOLINESTERASE AND NON-SPECIFIC CHOLINESTERASE IN THE RAT ADRENAL

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PREVIOUS studies have indicated that the adrenal medulla possesses an intense cholinesterase activity (ANTOPOL and GLICK, 1940; ORD and THOMPSON, 1952; KOELLE, 1950, 1951; ALLEN, ERÄNKÖ and HUNTER, 1958; COUPLAND and HOLMES, 1958). This is indeed what can be expected knowing that the secretory nerve impulses reach the adrenal medulla through cholinergic preganglionic fibres (FELDBERG, MINZ and TSUDZIMURA, 1934; HOLLINSHEAD, 1936; YOUNG, 1939; HILLARP, 1946; STÖHR, 1957). Recent histochemical work has shown that the distributions of acetylcholinesterase (AChE) and non-specific cholinesterase (nsChE) in the adrenal medulla of the rat somewhat differ from each other (ERÄNKÖ, 1958, 1959). The present work demonstrates that denervation causes a loss of AChE activity, but does not much affect the activity of nsChE in the rat adrenal.

METHODS

Experimental procedure. Adult healthy male albino rats were unilaterally denervated by dividing the left splanchnic nerve under the diaphragm. In the first experiments, the tissues above the left adrenal were also removed to make certain that the denervation was complete. Since this operation occasionally resulted in the appearance of necrotic areas in the adrenal, probably due to interference with the blood supply of the adrenal, it was later abandoned, and denervation was carried out by dividing the splanchnic nerve only. The changes observed with the present histochemical techniques were identical in both cases.

The number of denervated rats was 34. Four of them were killed 5–9 days, 17 rats 1–2·5 months and 13 rats 3–6 months after the denervation. Both adrenals were removed and sectioned with a freezing microtome at 20 μ, fresh or after fixation in calcium formol. The sections of the left, denervated, and right, intact, adrenals were floated on slides in pairs to guarantee identical treatment, the right adrenals serving as controls. These control adrenals were found to be histochemically identical, as far as AChE and nsChE are concerned, with adrenals from entirely untreated rats.

Histochemical techniques. Cholinesterases were demonstrated with KOELLE's (1951) improved method, using acetyl- and butyrylthiocholine as substrates. α-Naphtyl acetate was also used as a substrate, following the method given by PEARSE (1953), with the exception that a higher concentration of the coupling agent, diazotate of 5-nitroanisidine (Brentamine Fast Red B Salt, I.C I. Ltd.) was used (ALLEN, ERÄNKÖ and HUNTER, 1958).

Instead of di-*iso*propylfluorophosphate, as recommended by KOELLE (1951), 62.C.47 (1:5-*bis*-(4-trimethylammonium-phenyl)pentan-3-one di-iodide (BURGEN, 1949) and tetra-*iso*propylpyrophosphoramide (*iso*-OMPA) (ALDRIDGE, 1953) were used as inhibitors (PEPLER and PEARSE, 1957; ERÄNKÖ, 1958, 1959). In a concentration of 10^{-6} M, *iso*-OMPA completely abolished the reaction obtained with butyrylthiocholine and the eserine-sensitive component of the reaction with α-naphtyl acetate.

The reaction obtained with butyrylthiocholine and 10^{-5} M-62.C.47 was interpreted as due to nsChE activity. Essentially similar medullary distribution was also obtained with alpha-naphtyl

acetate and 10^{-5} M-62.C.47. The reaction obtained with acetylthiocholine in the presence of 10^{-6} M-*iso*-OMPA was ascribed to AChE. Incubation with both thiocholine substrates in the presence of 10^{-6} M-*iso*-OMPA and 10^{-5} M-62.C.47 resulted always in unstained slides. Incubation was carried out at 37°C for 1–1·5 hr. Before incubation in the substrate mixtures, the sections were pre-incubated with the same inhibitor(s) as used in the substrate mixture. For details see ERÄNKÖ (1959).

RESULTS

The results are summarized in Table 1, and illustrated by Figs. 1–4. Similar changes were observed in all animals regardless of the length of the period from the denervation. The AChE activity was radically affected by denervation. Almost all the medulla was entirely negative, the only reacting elements being some ganglion cells and the fibres originating from them, in which the reaction was strongly positive. Some fine cortical fibres reacted also after denervation.

TABLE 1.—EFFECT OF DENERVATION ON CHOLINESTERASES OF THE ADRENAL MEDULLA

Structure	Acetylcholinesterase		Non-specific cholinesterase	
	Control	Denervated	Control	Denervated
Capsule	−	−	+++	+++
Glomerulosa fibres	−	−	++	++
Cortical fibres	++	++	++	++
Nerve trunks	+++	−	+++	+++
Ganglion cells	+++	+++	+	+
—their fibres	++	++	+++	+++
—satellite cells	++	−	+++	++
Fine med. fibres	+++	−	−	−
Fibres around noradrenaline cells	+++	−	+++	++
Medullary cell cytoplasm	−	−	+	+

Although most of the AChE activity had disappeared, the activity of nsChE remained essentially unchanged. The only site where a slight change was observed was the nerve net associated with the noradrenaline-cell islets, in which the activity seemed to diminish somewhat. The distribution and intensity of the reaction was unchanged in all other structures which, in the normal rat adrenal, showed nsChE activity.

Since only some mid-sections were examined from each adrenal it was not possible to obtain a reliable idea of the quantitative proportions of different medullary tissues. However, it seemed that the number of nerve trunks and coarse nerve fibres was diminished in the denervated adrenals, although the nsChE reaction was unchanged in the persisting fibres.

DISCUSSION

In the present experiments denervation caused a pronounced loss in the AChE activity of the adrenal medulla. This change was evident 5 days after denervation and

was to be expected, if it is accepted that the medulla is innervated by cholinergic preganglionic fibres. Since the change produced by division of the splanchnic nerve alone was the same as that produced by division of this nerve and removal of peri-adrenal tissues, it is evident that at least a large majority of the cholinergic fibres of the rat adrenal medulla come from the splanchnic nerve. No signs of regenerating AChE positive fibres were seen even 6 months after denervation. This is of interest in view of the observation made by HOLLINSHEAD and FINKELSTEIN (1937) that, in the cat, the regeneration process is complete in about 4 months. Their inference that, in small laboratory animals, a permanent denervation would appear impossible to achieve, does not seem to apply to the rat.

The medullary ganglion cells and the fibres originating from them, as well as some fine fibres in the cortex, showed an AChE activity which was not affected by splanchnic denervation. This suggests that these structures are independent of the nerve fibres which reach the medulla through the splanchnic nerve and which, at least in the rat, seem to be responsible for the secretory innervation of the medulla. The significance of the medullary ganglion cells and the fine cortical fibres is, in spite of much study, still obscure; but the present work lends some support to the assumption that these nervous structures, which are also cholinergic, have functions other than the regulation of secretion from the adrenal medulla (see STÖHR, 1957).

The lack of changes in the activity of nsChE after denervation suggests that this enzyme is not located in the nerve fibres themselves and that it is not directly involved in the breakdown of the transmitter substance released during secretory stimulation of the adrenal medulla. It may be that this enzyme is located in the supporting nervous elements, (cf. KOELLE, 1955), a point which it has not been possible to verify with the technique used in the present work. It is somewhat surprising that the nsChE activity, which appears to be located in a fine network of fibres around the noradrenaline cell islets, is not destroyed by denervation.

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SUMMARY

After division of the splanchnic nerve, acetylcholinesterase activity disappears from the nerve trunks and fine medullary nerve net of the adrenal medulla. However, medullary ganglion cells and some fine cortical fibres retain their strong acetylcholinesterase activity after denervation. The activity and distribution of non-specific cholinesterase are practically unaffected by splanchnic denervation.

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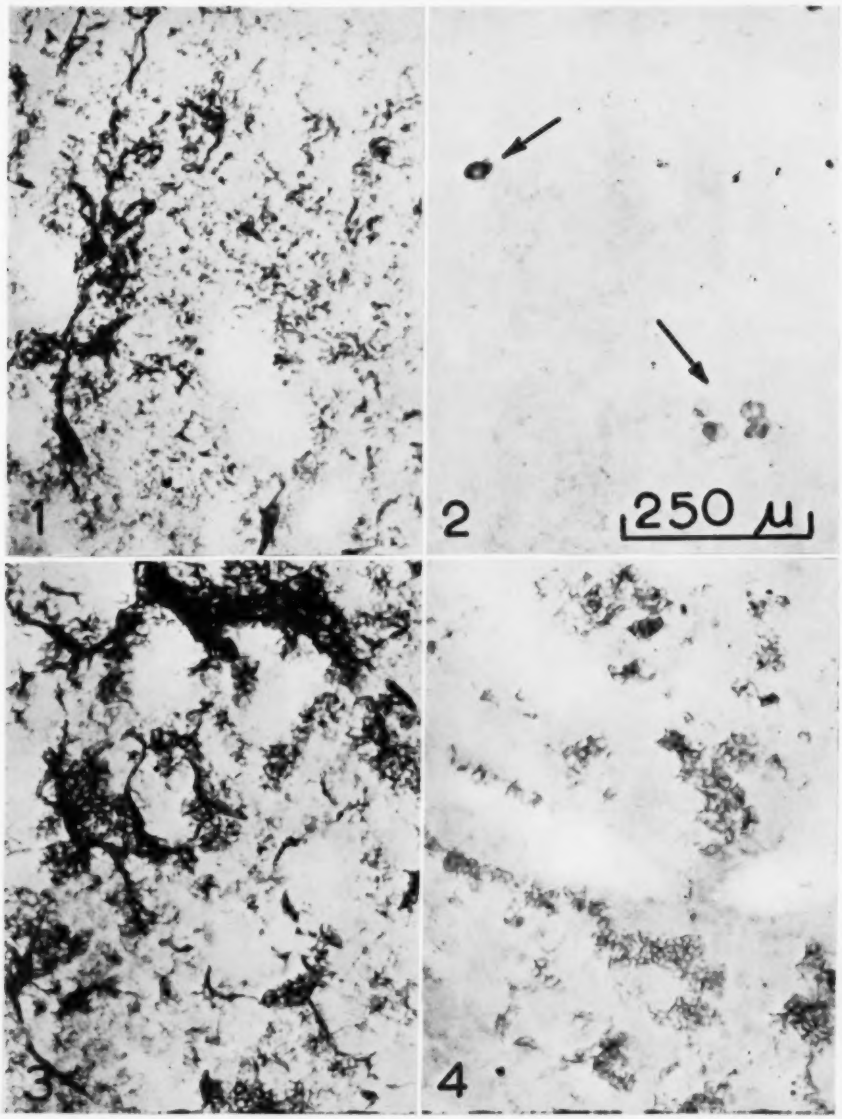

FIG. 1.—Control: AChE.

FIG. 2.—Denervated for 5 months. AChE. Arrows indicate ganglion cells.

FIG. 3.—Control: nsChE.

FIG. 4.—Denervated for 5 months. nsChE.

All figures are of the adrenal medulla of rats, ×100.

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ERRATUM

M. LEES, J. FOLCH, G. H. SLOANE STANLEY and S. CARR (1959) *J. Neurochem.* **4,** 9.

The captions for Figs. 3 and 4 should read as follows:

FIG. 3. Hydrolysis of sulphatide *A* by saturated $Ba(OH)_2$ at 100°.
——— Release of NH_2—N as estimated by the VAN SLYKE nitrous acid method (1929).
– – – – – Liberation of galactose. Since free galactose is destroyed by alkali, these figures were calculated from the amount of galactose which remained unhydrolysed.

FIG. 4. Release of inorganic sulphur from sulphatide *A* in the presence of glacial acetic acid at 100°.

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Papers to be published in future issues

R. VRBA and J. FOLBERGROVÁ: Observations on endogenous metabolism in brain *in vitro* and *in vivo*.

L. MAY, M. MIYAZAKI and R. C. GRENELL: The distribution of glutamic-oxaloacetic acid transaminase in rat brain.

V. BONAVITA: Purification and properties of glutamic-oxaloacetic transaminases from human brain.

C. W. M. ADAMS and A. N. DAVISON: The occurrence of esterified cholesterol in the developing nervous system.

L. FRIEDE: Histochemical investigations on succinic dehydrogenase in the central nervous system—III. Atlas of the midbrain of the guinea pig, including pons and cerebellum.

R. RYBOVÁ: The effect of cations on ammonia accumulation in brain cortex slices.

S. BERL, D. P. PURPURA, M. GIRADO and H. WAELSCH: Amino acid metabolism in epileptogenic and non-epileptogenic lesions of the neocortex (cat).

A. CARLSSON, E. B. RASMUSSEN and P. KRISTJANSEN: The urinary excretion of adrenaline and noradrenaline by schizophrenic patients during reserpine treatment.

A. CARLSSON, E. B. RASMUSSEN and P. KRISTJANSEN: The urinary excretion of adrenaline and noradrenaline by depressive patients during iproniazid treatment.

G. B. ANSELL and S. SPANNER: The effect of insulin on the formation of phosphorylcholine and phosphoryl-ethanolamine in the brain.

O. ERÄNKÖ, V. HOPSU and L. RÄISÄNEN: Changes induced by prolonged administration of nicotine in the distribution of cholinesterases and acid phosphatase in the adrenal medulla of the rat.

R. E. MCCAMAN and E. ROBINS: Quantitative biochemical studies of Wallerian degeneration in the peripheral and central nervous systems—I. Chemical constituents.

R. E. MCCAMAN and E. ROBINS: Quantitative biochemical studies of Wallerian degeneration in peripheral and central nervous systems—II. Twelve enzymes.

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M. B. SPORN, W. DINGMAN, A. DELFALCO and R. DAVIES: The synthesis of urea in the living rat brain.

J. AXELROD, W. ALBERS and C. CLEMENTE: Distribution of catechol-o-methyl transferase in the nervous system and other tissues.

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N. CANAL and A. MAFFEI-FACCIOLI: Reversal of the reserpine-induced depletion of brain serotonin by a monoamine oxidase inhibitor.

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THE DISTRIBUTION OF GLUTAMIC—OXALOACETIC ACID TRANSAMINASE IN RAT BRAIN*

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THE metabolism of glutamic acid is important in the functioning of the nerve cell. It is essential in understanding the role of glutamic acid to know the characteristics and distribution of those enzymes concerned with the synthesis and utilization of this amino acid. The enzyme, glutamic-oxaloacetic transaminase (GOT) which catalyses the formation of glutamic acid from α-ketoglutaric acid and aspartic acid, is an important link in the synthesis of glutamic acid. A knowledge of the concentrations of this enzyme in the different subcellular fractions should assist in localizing the synthetic mechanisms for the formation of glutamic acid. It is important to know the concentration of enzymes in the various brain areas as part of a chemical map from which the metabolic differences between cell groups can be evaluated. Thus localization becomes important at two levels; (a) the cell group and (b) the intracellular fractions. This investigation was undertaken to provide the necessary information about the distribution of GOT in the various areas and cellular fractions of the rat brain.

EXPERIMENTAL

Preparation of tissues. Analyses were performed on tissues removed from the brains of unanaesthetized, adult, white male rats (300–350 g). The animals received a stunning blow on the back of their heads and were then decapitated. When whole brain was used, the tissue was blotted with filter paper to remove excess blood before homogenizing with approximately 15 ml of ice-cold 0·25 M-sucrose. Dissection of the brain into the separate areas was done on filter paper. Where it is not otherwise specified, these samples were within anatomically demarcated brain areas and consisted of both grey and white matter. The tissue was blotted to remove excess blood and homogenized with ice-cold medium. The ratio of the volume of medium, V_s, to the wet weight of the tissue, W, was approximately 10 for all areas except hypothalamus and thalamus where V_s/W was 20–30. This was necessary because the tissue weights were 20–30 mg and a minimum volume of 0·5 ml was required.

All tissues were homogenized with a steel pestle and kept ice-cold during the homogenization. When the tissue extracts were to be analysed, the suspensions were centrifuged at 23,000 g for 15 min and clear supernatants saved for the analysis. Tissue homogenates were prepared in 0·25 M-sucrose.

Fractionation experiments. Whole rat brains were fractionated according to procedure II of BRODY and BAIN (1952) using 0·25 M-sucrose. The various fractions were designated in the same manner used by these authors. The residues from the first two centrifugings were combined and analysed (R_1R_2). The livers were fractionated in the manner described by HOGEBOOM (1955). The fractions are also described by the convention of WAKED and KERR (1955) in which the centrifugal force used to sediment a fraction is expressed in units (G) equal to 1000 × gravity (g) with the time expressed in minutes. Hence, 0·8 G 10 refers to that fraction obtained by centrifugation at 800 × g for 10 min.

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TABLE 1.—GOT IN DIFFERENT AREAS OF THE BRAIN
(μmoles DPNH oxidized/g wet tissue per hr)

Preparation analysed	No. of rats	Brain area							
		Cerebral cortex (grey)	White matter, (sub-cortical)	Cerebellum (whole)	Hypo-thalamus	Thalamus	Medulla	Midbrain	Pons
Homogenate	4	4420 ±70*	4410† ±140	4640 ±200	3980 ±250	4600 ±160	3960 ±70	4910 ±120	5130 ±180
Supernatant									
Phosphate buffer, pH 7·5	2	83	98	129	323	284	82	87	80
0·85% (w/v) NaCl	1	637	935	796	330	613	190	635	618
0·25 M-sucrose	3	1070	1170	1560	1120	960‡	1490	1310	1520

* Standard deviation.
† 3 rats.
‡ 2 rats.

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GOT analysis. The supernatants from the centrifugation of the homogenates and the homogenates were stored in the deep-freeze until the time of analysis. The samples were brought to 25° in a thermostat before the reagents were added. The procedure for the GOT analysis is the same as used by KARMEN, WROBLEWSKI and LADUE (1955). The reaction was followed in a Beckman Model B Spectrophotometer at room temperature (25°). The temperature of the reacting mixture did not vary more than 0·5° during the spectral readings. The enzymatic activity is expressed as μmoles DPNH oxidized/g wet tissue/hr (WAKIM and FLEISHER, 1956). The reproducibility of the analyses was $\pm$10 per cent. The samples were diluted so that the change in absorbance at 340 mμ in 5 min was 0·050–0·125.

RESULTS

Previous investigators had measured GOT in brain extracts so that initially, different media were examined to determine their efficacy in extracting GOT. Thus, homogenate preparations were made as described above. After centrifuging, the activities were measured in the supernatants. When these values were later compared with the GOT yields from whole homogenate preparations, it became apparent that only a small percentage of the total activity was in the supernatant. It was established that most of the enzymatic activity remained in the homogenate residue after extraction and centrifugation. The activities found in the various extracts and homogenates of the different areas of the brain are given in Table 1. The values for the activities found with the various extracting agents are presented to provide a basis of comparison with previous results reported in the literature. It is apparent that a satisfactory yield is only obtained with the whole homogenate preparations. It should be noted that of the three extracting agents used, 0·25 M-sucrose appeared to be the best. The relative amounts extracted from a particular area varied with the extracting agent. This indicates that these agents vary widely in the efficiency of their removal of this protein in different areas. The results obtained with homogenates indicate that the order of GOT activity/g wet tissue decreases in the following order: pons, midbrain, thalamus, cerebellum, cerebral cortex, white matter, medulla and hypothalamus.

The whole brain was separated into the different cellular fractions by centrifuging and each fraction assayed for GOT (Table 2). For comparison, analyses were also performed on rat liver fractions (Table 2). In both tissues, GOT is found distributed equally in the mitochondrial (R_3) and nuclear (R_1R_2) fractions. About half of the amount found in R_3 is located in the supernatant fraction.

The pH-activity curve of GOT in whole brain extracts (0·25 M-sucrose) was found to be similar to the one previously reported for blood.

KARMEN *et al.* (1955) incubated the mixture containing blood, DPNH, and aspartic acid for 10 min prior to the addition of α-ketoglutaric acid. This was necessary to permit the indigenous DPNH-utilizing systems to act without interfering with the GOT analysis. This period of time has been found to be sufficient with the tissues examined in the present investigation.

The GOT activity of homogenates of whole brains removed from three rats which had been injected with chlorpromazine (50 mg/kg) was 5240 $\pm$ 860 μmoles DPNH oxidized/g wet tissue/hr. The difference between this value and the activity found in control animals is not statistically significant.

DISCUSSION

Most of the GOT analyses previously reported on brain were obtained using various extracting media, methods and whole brain (AWAPARA and SEALE, 1952;

TABLE 2.—GOT IN FRACTIONS OF BRAIN AND LIVER
(μmoles DPNH oxidized/g wet tissue per hr)

No.	Homogenate	R_1R_2		R_3		R_4		S		Total recovery
		Nuclei, cell debris		Mitochondria		Microsomes		Supernatant		
	GOT	GOT	%	GOT	%	GOT	%	GOT	%	%
				Whole Brain						
		0·8 *G* 100 and 1·5 *G* 10		12 *G* 15		23 *G* 30				
(1)	4000	2120	50	1200	30	43	1	680	17	101
(2)	4980	2100	42	1410	28	54	1	672	14	85
(3)	3940	2340	60	1620	41	45	1	630	16	117
Mean	4310 ± 580*									
				Whole Liver						
		0·7 *G* 10		24 *G* 10		54 *G* 60				
(1)	5920	2130	36	2410	41	240	4	860	15	95
(2)	6350	—	—	3180	50	108	2	1210	19	—
(3)	7250	2130	29	2080	29	144	2	609	8	69
Mean	6510 ± 680*									

% (GOT in fraction/GOT in homogenate) 100.
* Standard deviation.

COHEN, 1951). BRAUNSTEIN (1947) stated that the cortex contains more transaminase than white matter. WAKIM and FLEISHER (1956) measured GOT in several areas of dog brain and confirmed this observation. This differed from the results obtained in the present investigation (Table 1) where tissue homogenates were used. The amount of GOT in both grey and white matter is the same when homogenates are analysed. This may be due to differences in the purity of the samples as well as to the presence of glial cells in the white matter. It is interesting to note that when 0·85% saline is used as an extracting agent, the medullary level of GOT is lower than that of the cerebral cortex (Table 1, and WAKIM and FLEISHER, 1956), but the values are reversed when sucrose is used as the extractant. Other areas show different changes from medium to medium. The results of different investigators cannot be compared because different extracting solvents, different temperatures during the analyses, and various species were used. LOWRY (1957) has reported a few values for GOT obtained by micro-methods using whole rabbit brain homogenates and several special cell groups.

The effect of varying the extracting medium is to vary the relative amounts of GOT extracted from the different areas. This is related to relative efficiences of the

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TABLE 3.—GOT IN BRAIN AREAS
(μmoles DPNH oxidized/hr)

Area	GOT (activity/g wet tissue)	DNAP* (μg/g wet tissue)	GOT† (activity/cell)
Cerebellum	4640 ± 200‡	382 ± 40	0·79 ± 0·09
Thalamus	4600 ± 160	83 ± 11	3·60 ± 0·49
Cerebral cortex	4420 ± 70	70 ± 6	4·10 ± 0·36
White matter	4410 ± 140	84 ± 11	3·40 ± 0·46
Hypothalamus	3980 ± 250	103 ± 24	2·50 ± 0·61
Medulla	3960 ± 70	94 ± 24	2·70 ± 0·70

* DNAP—Deoxyribonucleic acid phosphorus, MAY and GRENELL (1958).

† $\dfrac{\text{GOT activity} \times 10^{-5}}{\text{cell (nucleus)}} = \dfrac{\text{GOT activity/g wet tissue}}{\text{cells/g wet tissue}}$

where $\dfrac{\text{cells}}{\text{g wet tissue}}$ is taken as $\dfrac{65 \times 10^{-8}\,\mu\text{g DNAP§/cell}}{\mu\text{g DNAP/g wet tissue}}$

‡ Standard deviation.

§ GRAY and DELUCA (1956).

solvents in extracting this protein from brain tissues. Since pyridoxal phosphate is the coenzyme for GOT (COHEN, 1951) the effect of the different solvents on the extraction of this compound and the binding between the protein and its coenzyme should be considered.

Homogenates contain all the pyridoxal phosphate and the protein moiety of GOT present in the tissue. The results obtained in this investigation indicate that the use of homogenates gives reliable values of the GOT activity in the tissue.

The values of GOT activity reported on the basis of activity/g wet tissue fall into three groups (Table 1): (1) pons and midbrain; (2) cerebellum, thalamus, cerebral cortex and white matter; and (3) hypothalamus and medulla. If the average activity per cell is calculated, the activities of the areas fall into different groupings (Table 3). The calculation of the average GOT per cell is based upon the assumption that the concentration of deoxyribonucleic acid (DNA) per nucleus is constant in all cerebral cells (see discussion of NURNBERGER and GORDON, 1957). An average value of 65×10^{-8} μg DNAP per nucleus is used in these calculations (GRAY and DELUCA, 1956). The values for DNAP/g wet tissue were obtained using the ultraviolet spectrophotometric method of LOGAN, MANNELL and ROSSITER (1952). Cerebellar tissue contains the least average activity per cell whereas cortical tissue contains the most. Values for pons and midbrain were not included because DNAP concentrations in these areas were not available.

The results of the fractionation of both brain and liver indicate that the distribution is the same in both tissues (Table 2). GOT is equally distributed in the R_1R_2 and R_3 fractions and about half of the activity in these fractions is found in the supernatant. It is difficult to assess the origin of the activity in R_1R_2 (cell debris and nuclei), and no conclusions can be made about the activity in the nuclei. As with other published studies in which fractionation was carried out by these procedures, the possibility always exists that the fractions are not pure. It is planned to check their purity

after more refined fractionation procedures have been employed. Since the liver fractionation procedures are well established as being adequate for this tissue, it is of interest to compare the results of brain and liver fractionations by the same simple methods.

SUMMARY

Glutamic–oxaloacetic acid transaminase (GOT) analyses were made with tissue homogenates which were found to contain about four times the activity found in tissue extracts.

The activity in the several areas of the rat brain was found to fall into three groups expressed as GOT/g wet tissue; (1) pons and midbrain; (2) cerebellum, thalamus, cerebral cortex and white matter; and (3) hypothalamus and medulla. A different order is found when the activity is calculated on the basis of GOT per cell. Cerebral cortex then is most active and cerebellum the least.

Rat whole brain and liver were fractionated by centrifugation. Both tissues showed the same fractional GOT distribution. Approximately the same activities were found in the mitochondrial (R_3) and the nuclear (R_1R_2) fractions. About 15 per cent of the GOT activity was recoverable in the supernatant.

The GOT activity in whole brain homogenates isolated from rats treated with chlorpromazine was not significantly different from the activity of whole brain homogenates of control animals.

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PURIFICATION AND PROPERTIES OF GLUTAMIC–OXALOACETIC TRANSAMINASE FROM HUMAN BRAIN

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GLUTAMIC-oxaloacetic transaminase (GOT) is found in a wide variety of tissues. GOT from pig heart has only recently been obtained in a highly purified form by JENKINS and SIZER (1957) and by LIS (1958). Many properties of this enzyme have been described (BRAUNSTEIN, 1947; COHEN, 1951; MEISTER, 1955), but no procedure is available for the purification of the enzyme from brain.

The present paper deals with GOT obtained in purified but not crystalline form from the human brain. Extraction and purification procedures are described, as well as the kinetics of the enzyme under different conditions. Absorption spectra of the enzyme are also given. Some, but not all the properties of the enzyme from human brain are similar to those of the enzyme prepared from pig heart.

EXPERIMENTAL

Preparation of the enzyme. Table 1 summarizes the purification procedure. A human brain weighing 1000 g was washed in saline (0·15 M-NaCl) and the arachnoid and pial meninges were

TABLE 1.—PURIFICATION OF GLUTAMIC-OXALOACETIC TRANSAMINASE FROM HUMAN BRAIN

Procedure	Vol. (ml)	Activity without Py5P (units/ml)	Activity with Py5P (units/ml)	Total units (without Py5P)	Total units (with Py5P)	Protein (mg/ml)	Purity (units/mg) without Py5P	Purity (units/mg) with Py5P
(1) Initial extract after centrifugation at 32,000 g	1100	126	130	138,000	142,000	—	—	—
(2) After heating	1000	56	118	56,000	118,000	10·0	5·60	11·80
(3) After butyl alcohol	940	66	138	66,000	129,500	9·0	7·35	15·40
(4) After alumina	940	48	105	45,000	99,000	5·5	8·70	19·10
(5) After elution from calcium phosphate gel	194	146	238	28,500	46,200	10·0	14·60	23·80
(6) After ammonium sulphate and dialysis	26	650	1620	16,900	42,200	40·0	16·20	40·50
(7) After chromatography, ammonium sulphate and dialysis	3	5200	13,200	15,600	39,600	4·1	1268	3219

Values refer to transamination between L(+)aspartic and 2-oxoglutaric acid. Temperature: 37°.
Py5P = pyridoxal-5-phosphate. 1 unit = 0·001 change in o.d. at 280 mμ in 1 min.

removed. The brain was cut in small fragments and homogenized in 1·5 l. of 0·05 M-sodium maleate buffer, pH 6, for 1 min in an 'Omni-Mixer Servall' (Ivan Sorvall, Inc.). Subsequent extraction was carried out at 4° with mechanical stirring for 12 hr. The thick superficial foam was removed, and the homogenate was centrifuged in a Spinco ultracentrifuge (model L) for 30 min at 32,000 g (step 1). The supernatant was heated for 5 min in a water bath held at 52° (step 2). After rapid cooling, it was centrifuged as before, and *n*-butanol was added slowly with vigorous agitation until a 5% concentration (v/v) was reached, according to the method by MORTON (1955). The cloudy suspension was heated at 37° for 3 min (step 3). Another centrifugation was carried out for 45 min at 32,000 g, and alumina, prepared according to HAWK, OSER and SUMMERSON (1947), was added to the supernatant to give a final concentration of 10 per cent (v/v) (step 4). After standing 20 min at 4°, the suspension was centrifuged, and 15 vol. % of calcium phosphate gel, prepared according to KEILIN and HARTREE (1937–8), were added. After 30 min, another centrifugation was performed, and the supernatant was decanted. The calcium phosphate gel was washed three times with distilled water, and the enzyme was eluted with 0·067 M-$Na_2HPO_4 \cdot 2H_2O$, plus $(NH_4)_2SO_4$ (10%, w/v) (step 5). To 100 ml of the eluate, 48 g of ammonium sulphate were added, and after 30 min, the suspension was centrifuged and the precipitate was dissolved in 15 ml of 0·3 M-sodium maleate buffer, pH 6, containing 0·0025 M-2-oxoglutaric acid, as suggested by JENKINS and SIZER (1958) (step 6).

Dialysis against distilled water was carried out for 12 hr with constant stirring. The material was centrifuged and then subjected to column chromatography on hydroxyl-apatite according to the method of TISELIUS (1956), under the experimental conditions prescribed by JENKINS and SIZER (1958) for purifying GOT from pig heart. 2-Oxoglutaric acid (final concentration, 0·0025 M) was added to fractions containing the enzyme, and the mixture was fractionated with ammonium sulphate. The addition of ammonium sulphate is stopped just when the solution becomes cloudy (usually when added ammonium sulphate exceeds 35 g per cent). After centrifugation, 5·5 g of ammonium sulphate were added to 100 ml of enzyme solution. The precipitate was dissolved again in 0·3 M-sodium maleate buffer, pH 6, and dialysis was carried out with numerous changes of distilled water for 24 hr (step 7). Traces of haem were removed by a further fractionation with ammonium sulphate, as was done in the purification of GOT from pig heart (JENKINS and SIZER, 1958).

Absorption spectra of the enzyme. Absorption spectra of the purified GOT were determined between 280 and 450 mμ with a Beckman DU spectrophotometer. The enzyme was diluted with 0·1 M-sodium acetate buffer, 0·1 M-sodium phosphate buffer, and 0·1 M-glycine–NaOH buffer at pH values between 4·8 and 9·0.

Activity tests. The preparation was controlled at each purification step by measuring the extinction increase or decrease at 280 mμ due to formation or disappearance of oxaloacetic acid, under the experimental conditions prescribed by CAMMARATA and COHEN (1951). The assay technique described by TONHAZY, WHITE and UMBREIT (1950) was used with L(+)alanine as a substrate.

pH-activity curves. A 0·05 M-sodium phosphate buffer was used. The pH values were measured at the end of the reaction with a glass electrode.

Experiments with acid phosphatase. Acid phosphomonoesterase was purified from human prostate according to VESCIA and TESTI (1958): 1 mg of protein, under the experimental conditions described by VESCIA (1957), splits 0·6 m-moles of sodium monophenylphosphate in 1 min. Before measuring transaminase activity, 25 μg of acid phosphatase were added to 20 μg of GOT and incubated at 37° for 10 min in 0·1 M-sodium acetate buffer, pH 5·2.

Experiments with inhibitors. The enzyme preparation was incubated with different concentrations of the compounds examined, after incubation of the holoenzyme with L(+)aspartic acid. The molarities given in Fig. 6 are those existing during the incubation of the inhibitors with the enzyme system (10 min). The pH of incubation mixtures was 7·1. The enzymic activity was measured after 1 : 3 dilution. In the case of phenylmercuric acetate, the holoenzyme was incubated with the inhibitor before the addition of L(+)aspartic acid.

Reagents. The following compounds were obtained from Hoffmann-La Roche (Switzerland): L(+)aspartic acid, L(+)glutamic acid, L(+)alanine, L(+)glutamine, L(+)asparagine, 2-oxoglutaric acid, D-*cyclo*serine, iproniazid (Marsilid), pyridoxal-5-phosphate. Oxaloacetic acid, from Fluka (Switzerland); crystalline isoniazid (Nicotibina), from Lepetit (Italy); D,L-penicillamine, from Aldrich (U.S.A.); maleic acid, from Bayer (Germany); succinic acid and sodium monophenylphosphate, from Merck (Germany); phenylmercuric acetate and iodoacetic acid, from Light & Co (England); and pyridoxamine-5-phosphate, from Siti (Italy).

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RESULTS

Absorption spectra. The ultraviolet absorption spectrum of GOT purified from human brain is influenced by pH in the range between 4·8 and 9, in a way similar to that observed for GOT from pig heart by JENKINS and SIZER (1957), but the pK'_a which was found by JENKINS and SIZER at pH 6·2 for pig heart GOT, was found at pH 5·7 for the present enzyme. Mathematical analysis gave $n = 1$, and it seems possible to try to interpret the pK'_a value determined in this investigation following the suggestions of those authors.

The addition of L(+)aspartic acid causes a shift of the extinction maximum from 360 to 330 mμ, at pH 7·6 (Fig. 1). It must be noted that the spectrophotometric

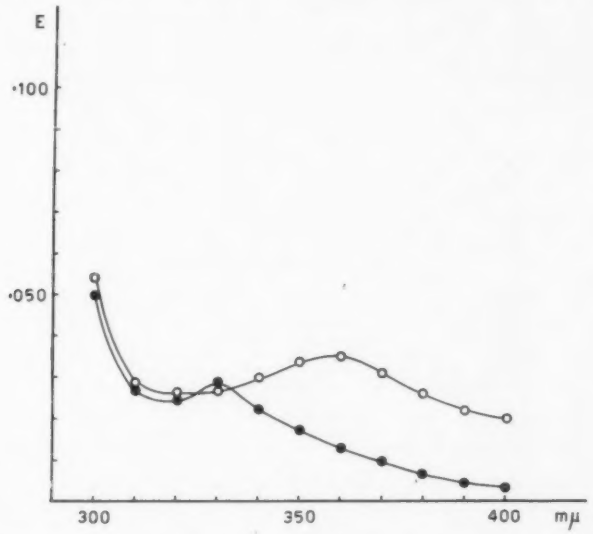

FIG. 1.—Absorption spectra of GOT from human brain in 0·1 M-sodium phosphate buffer, pH 7·6, before (○) and after (●) addition of L(+)aspartic acid. Experimental conditions: 0·2 mg protein and 0·02 m-moles (L+)aspartic acid in 1 ml final volume.

analysis of our preparation was difficult owing to the partial resolution of the enzyme.

Dependence of the transaminase activity on the enzyme concentration. The velocity is proportional to the enzyme concentration when L(+)aspartic acid and 2-oxoglutaric acid are both present in excess. This was shown by plotting the change in optical density at 280 mμ in 30 sec at 37° (ΔE_{280}) against any protein contained in 3·2 ml. A straight line was obtained only when the protein concentration in the final volume ranged from 0·02 to 0·08 mg, and the substrate concentration was of 20 μmoles.

pH-activity curves. Dependence of enzyme activity on pH is shown in Fig. 2. The pH optimum lies well within physiological pH values, and it is the same for both the reactions catalysed by GOT.

Substrates. The velocity of the reaction varied with substrate concentration. At pH 7·1, the K_m values obtained with L(+)aspartic acid and L(+)glutamic acid were $1{\cdot}67 \times 10^{-3}$ M and $1{\cdot}82 \times 10^{-3}$ M, respectively. They do not agree with the K_m value by COHEN (1951) for GOT from another source ($1{\cdot}38 \times 10^{-2}$ M). L(+)alanine, L(+)glutamine and L(+)asparagine are not substrates.

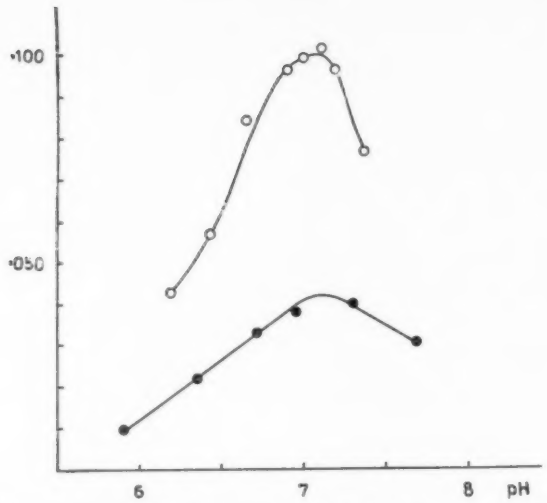

FIG. 2.—(A) Rate of transamination between L(+)aspartic acid and 2-oxoglutaric acid as a function of pH. Experimental conditions: 40 μg of protein and 20 μmoles of substrate in a final volume of 3·2 ml. Ordinates: increase in optical density at 280 mμ in 60 sec. Temperature: 25° (○).

FIG. 2.—(B) Rate of transamination between L(+)glutamic acid and oxaloacetic acid as a function of pH. Experimental conditions: 20 μg of protein and 3 μmoles of substrate in a final volume of 3·2 ml. Ordinates: decrease in optical density at 280 mμ in 30 sec. Temperature: 25° (●).

Experiments with coenzymes. The apparent K_m found for the interaction of the apo-enzyme with pyridoxal-5-phosphate is $4{\cdot}2 \times 10^{-6}$ M. This value is very close to that found by MEISTER, SOBER and PETERSON (1954) for GOT from pig heart ($4{\cdot}4 \times 10^{-6}$ M). Pyridoxamine-5-phosphate did not activate the apo-enzyme even after incubation with it for 90 min at 37°.

Thermodynamic analysis of the reaction. The value of the energy of activation was calculated from the slope of the Arrhenius plot of data on transamination between L(+)aspartic acid and 2-oxoglutaric acid: it was found to be 5960 cal/mole over the temperature range studied (25°–40°C). This value does not agree with the value found, under different experimental conditions, by NISONOFF and BARNES (1952) for GOT from pig heart (12,500 cal/mole).

Experiments with acid phosphatase. As pyridoxal-5-phosphate is hydrolysed by acid phosphatase (BONAVITA, 1958), some experiments were carried out in order to test the action of this enzyme on the partially resolved GOT molecule. It was found that this treatment did not affect the ultraviolet absorption spectrum or the enzymic activity of GOT. When bound to apo-transaminase, pyridoxal-5-phosphate is not attacked by acid phosphomonoesterase from human prostate.

Experiments with inhibitors. Different compounds were tested for their effects on GOT. There was no inhibition with up to 10^{-3} M-isoniazid. A similar finding was reported for alanine-2-oxoglutaric acid transaminase from pig heart by HICKS and CYMERMAN-CRAIG (1957). It was also found that iproniazid ($1{\cdot}5 \times 10^{-3}$ M) and D,L-penicillamine did not inhibit GOT activity.

Sodium succinate inhibited by only 15 per cent at a concentration of $1{\cdot}42 \times 10^{-2}$ M, while sodium maleate acted as a more potent inhibitor, as was already observed by

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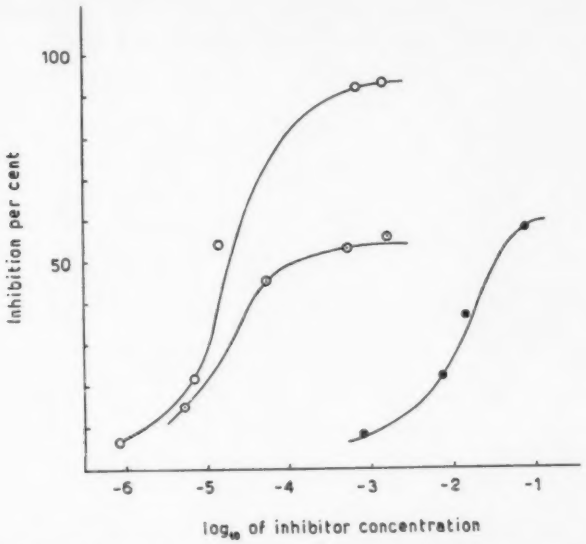

FIG. 3.—Per cent inhibition of transamination between L(+)aspartic acid and 2-oxoglutaric acid as a function of the logarithm of inhibitor concentration. Experimental conditions: 25 μg of protein and 20 μmoles of substrate in a final volume of 3·2 ml. Buffer: 0·05 M-sodium phosphate, pH 7·1. Temperature : 37°. D-*cyclo*serine (○); phenylmercuric acetate (⊙); sodium maleate (●).

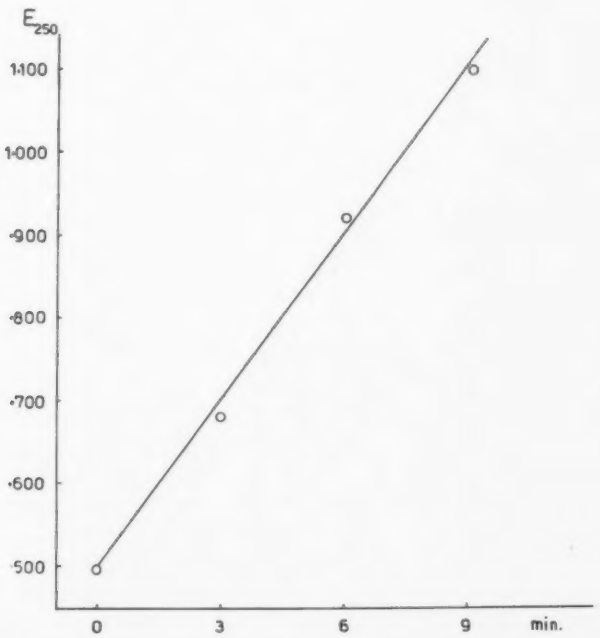

FIG. 4.—Rate of the reaction between the enzyme protein and phenylmercuric acetate. Experimental conditions: 150 μg of protein and 6 μmoles of phenylmercuric acetate in a final volume of 3 ml. Buffer: 0·05 M-sodium phosphate, pH 7·1. Temperature: 37°. Ordinate: change in optical density at 250 mμ (see BOYER, 1954).

JENKINS and SIZER (1957) for GOT from pig heart. D-*Cyclo*serine produced the greatest inhibition of all compounds tested. Phenylmercuric acetate produced a definite inhibition (Fig. 3). The results shown in Fig. 4 demonstrate the formation of a mercaptide between phenylmercuric acetate and the enzyme protein. Sodium iodoacetate was without effect.

DISCUSSION

The purification procedure deserves some comment. It seems that in human brain the coenzyme is less firmly bound to the apo-transaminase than in pig heart. Although it is more drastic, the procedure of JENKINS and SIZER (1958) for purifying GOT from pig heart achieves much less resolution than does the present technique applied to GOT from brain.

The specific activity of GOT from human brain was 20·7 (μmoles of oxaloacetic acid formed per 1 mg protein per minute, at 37°). This value is definitely lower than that observed by JENKINS and SIZER (1957) for the enzyme from pig heart. This discrepancy may be due to an insufficient purification of the brain GOT. It is possible on the other hand, that brain GOT has a lower specific activity than the enzyme from pig heart. Attempts to obtain a preparation of a higher specific activity were unsuccessful. However, since GOT cannot be completely reactivated after resolution (CAMMARATA and COHEN, 1951), the value reported above for the specific activity of GOT in human brain may well be lower than the true value.

The pH optimum for human brain GOT activity was found to be at pH 7·1. In this respect, GOT from human brain differs from the enzyme of other tissues. It may be possible to correlate the difference in pH optima with the difference between the pK'_a values of GOT from human brain and pig heart.

Experiments with pyridoxamine-5-phosphate yielded results of some interest. MEISTER *et al.* (1954) were able to activate apo-transaminase from pig heart through incubation with phosphopyridoxamine. This was not observed in the case of the brain enzyme. However, we believe that the lack of activation by phosphopyridoxamine does not contradict the well known scheme of SCHLENK and FISHER (1947) for the mechanism of transamination reactions. The presence in pyridoxamine-5-phosphate of an amino group with pK'_a value of 10·92 (WILLIAMS and NEILANDS, 1954) may explain the lack of activation by this compound as observed in the present study and by others (O'KANE and GUNSALUS, 1947). Moreover, it is possible that different alterations take place on the apo-protein molecule, according to the purification procedures applied.

The effects of inhibitors will now be considered. The inhibiting effect of phenylmercuric acetate indicates the presence on the GOT molecule of thiol groups capable of forming mercaptides. Since sodium iodoacetate was not an inhibitor, it may be concluded that, although thiol groups are essential for the activity of the enzyme, they are differently accessible to these two reagents.

The inhibition caused by D-*cyclo*serine is of some interest, since it is known to act as a convulsive agent and because of its ability to chelate metallic ions (NEILANDS, 1956). PATWARDHAN (1958) recently published a preliminary report on the role of ferrous iron in enzymatic transamination, but it is to be pointed out that the binding of ferrous iron itself by D-*cyclo*serine is extremely weak. On structural grounds, a reaction of D-*cyclo*serine with phosphopyridoxal is very probable.

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SUMMARY

(1) Glutamic-oxaloacetic transaminase from human brain was partially purified.
(2) A study of the kinetics and a thermodynamic analysis of the reaction catalysed by this enzyme are reported.
(3) Several compounds were tested as inhibitors.

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THE OCCURRENCE OF ESTERIFIED CHOLESTEROL IN THE DEVELOPING NERVOUS SYSTEM

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ESTERIFIED cholesterol, in relatively large amounts, has been found in the brain of developing chickens by MANDEL, BIETH and STOLL (1949). The presence of these esters in the young animal and their apparent absence (SPERRY, 1955) in the adult led us to postulate that esterified cholesterol may play an important role in the development of the central nervous system.

This paper describes histochemical observations on the developing CNS of man and chickens, together with parallel chemical and chromatographic determinations of cholesterol and its esters. Evidence is presented to show that part of the ester cholesterol is located in the newly formed myelin sheath. The relevance of these histochemical and chemical observations to the process of myelinogenesis is also discussed.

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MATERIALS AND METHODS

Fowl eggs were incubated at 37° and at certain stages the cord and brain were removed from the embryo or chick. Human brain (corpus callosum) and cervical cord were obtained either at autopsy or from therapeutic abortion.* Before dissection the corpses were stored at 4°. After dissection material for chemical determinations was kept at −20°, while samples for histochemical investigation were usually fixed in neutral 4% formaldehyde–saline.

Chemical methods. Cholesterol and its esters were determined in duplicate on nervous tissue as described by DAVISON, DOBBING, MORGAN and PAYLING WRIGHT (1958) with one modification: potassium hydroxide neutralized with acetic acid was added to the samples of unhydrolysed extract for the determination of free cholesterol. Triglyceride was determined by hydrolysing dried samples (0·25–1 mg) with 0·1 ml 25% (w/v) alcoholic KOH for 30 min at 100°. The alkaline hydrolysate was made acid with H_2SO_4 and the volume adjusted to 1 ml with water. The solution was shaken with 2 ml of chloroform and 0·4 ml of the aqueous layer was removed and its glycerol content was determined by the method of BURTON (1957).

Sterols were detected on paper by heating paper chromatograms dipped in 8% (w/v) phosphotungstate (DOUSTE-BLAZY, POLONOVSKY and VALDIGUIE, 1952). Small quantities of cholesterol and its esters (10–60 μg) in 10 ml eluates from the alumina column were estimated by the quantitative Tschugaeff reaction as described by HANEL and DAM (1955).

Column chromatography. Samples of brain or spinal cord were homogenized in 20 times their volume of chloroform : methanol (2 : 1 v/v) in a glass homogenizer. The suspension was filtered and portions were removed for analysis and a suitable volume containing about 1 mg of total cholesterol was concentrated to dryness and then taken up in 5 ml of petroleum ether. A column (1 × 20 cm) containing 10 g of alumina (BDH) was deactivated by the method of SCHÖN and GEY (1956). The lipid extract was added to the column which was then successively eluted with 50 ml of 1%, 70 ml of 3% and 80 ml of 30% (v/v) ether in petroleum ether (b.p. 50–60°). Fractions were collected for each of the three solvent mixtures.

Histochemical methods. Samples of the tissues used for chemical analysis were fixed in 4% formaldehyde–saline and cut on the freezing microtome at 15 μ. A few unfixed samples were embedded in unfixed liver and cut on the cryostat at 5 μ: these tissues were subsequently fixed in formalin.

* We are indebted to Dr. H. E. M. Kay of the Royal Marsden Hospital for this material.

The sections were stained by the following methods:—

Osmium tetroxide–α-naphthylamine–alcian blue (OTAN: ADAMS, 1958, 1959). Hydrophobic lipids (cholesterol ester and neutral fat) are stained black while hydrophilic lipids (phospholipid and mixtures containing phospholipid) are stained red.

Acid haematin (BAKER, 1946, 1947). Phospholipids are stained blue-black.

Ferric hydroxamate (ADAMS and DAVISON, 1959). Phosphoglycerides are stained orange.

Nile blue sulphate. Neutral fat (triglyceride) and to a lesser extent cholesterol esters are stained in shades of mauve.

Sudan black and Sudan IV.

Schultz reaction for cholesterol. This histochemical modification of the Lieberman–Burchard method was found to be too destructive to the tissues and to cause too much diffusion of the reaction product for accurate localization.

Lipid extraction. Extraction was carried out for 18 hr on sections which had been fixed on to chrome-gelatined slides. The solvents used were: chloroform–methanol (2 : 1 v/v) at 20°, acidified chloroform methanol (2% HCl) at 20°, acetone at 4° and pyridine at 20°.

RESULTS

Chemical estimations

Effect of post-mortem storage. A study was made of the effect of different kinds of storage on the preservation of cholesterol esters in the brain. This was necessary since most human material had been kept for some time both before and after autopsy. Random samples from the corpus callosum of a 2 month old child were stored for corresponding intervals at room temperature, at −20° and in formalin. Although results varied from one sample to another, duplicate analyses of individual samples were in excellent agreement. There was no significant difference between the means of ester in fresh samples and those stored for more than a week (fresh 9·1 ± 3·65; stored 11·8 ± 1·9 mg/g wet weight; $P = 0{\cdot}9$).

Separation and estimation of cholesterol esters by chromatography. The results of chromatography of a synthetic mixture of cholesterol, cholesterol oleate and

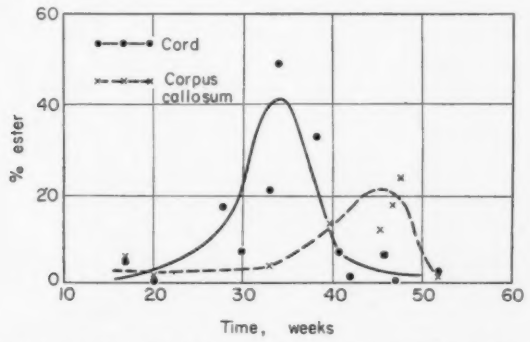

FIG. 1.—Cholesterol ester ratio in human cord and corpus callosum at different ages before and after birth.

triolein and typical results obtained with extracts of human brain and degenerating cord are presented in Table 1. Cholesterol esters were eluted from the column by the first solvent mixture and, accordingly, could then be estimated with greatly improved accuracy. Triglycerides were found in the second solvent fraction and free cholesterol in the last. The chemical analyses on fractions separated by chromatography always confirmed those analyses carried out on the crude extracts (Table 1).

TABLE 1.—CHROMATOGRAPHIC SEPARATION

Samples were prepared from human material and separated as described in the text. Cholesterol ester is expressed in terms of its cholesterol content. The synthetic mixture contained cholesterol oleate, triolein and free cholesterol. Free and esterified cholesterol and triglyceride was determined on the separated fractions and on crude extracts before fractionation. Solvents A, B and C were 1%, 3%, and 30% ether in petroleum ether respectively.

Sample		Weight extracted (mg)	Cholesterol as ester (mg)		Triglyceride (mg)		Free cholesterol (mg)	
			Fraction	Crude extract	Fraction	Crude extract	Fraction	Crude extract
Synthetic mixture	A	1·52	0·52	0·54	0·00	0·50	0·00	0·48
	B		0·02		0·45		0·14	
	C		0·00		0·05		0·40	
Infant corpus callosum (47 wk.)	A	39·9	0·09	0·088	0·00	—	0·00	0·18
	B		0·00		0·00		0·00	
	C		0·00		0·00		0·16	
Corpus callosum (3 year)	A	33·2	0·00	0·04	—	—	0·00	0·50
	B		0·00		—		0·00	
	C		0·04		—		0·50	
Corpus callosum (5 year)	A	33·3	0·00	0·04	—	—	0·00	0·76
	B		0·00		—		0·00	
	C		0·05		—		0·76	
Degenerating cord	A	57·2	0·35	0·42	0·00	—	0·00	0·97
	B		0·12		0·05		0·60	
	C		0·00		0·15		0·19	
Degenerating cord	A	38·6	0·28	0·28	—	—	0·00	0·527
	B		0·00		—		0·00	
	C		0·00		—		0·66	

TABLE 2.—ESTER AND FREE CHOLESTEROL LEVELS IN THE DEVELOPING CNS OF THE HUMAN

Age	Cord			Corpus callosum		
	Ester cholesterol (mg/g)	Free cholesterol (mg/g)	% ester	Ester cholesterol (mg/g)	Free cholesterol (mg/g)	% ester
Foetal (weeks)						
17	0·23	3·7	5·8	0·21	3·2	5·9
20	0·00	2·9	0·0	0·00	3·2	0·0
28	1·4	6·0	18·8	—	—	—
30	0·52	6·5	7·3	—	—	—
33	1·3	4·8	20·8	0·28	8·7	3·1
34	2·5	2·7	49·0	—	—	—
38	4·8	9·7	32·7	—	—	—
40	—	—	—	1·2	7·6	13·4
Infant (weeks)						
1	0·87	10·9	7·6	—	—	—
2	0·31	17·3	1·7	—	—	—
6	1·00	14·3	6·5	1·5	6·5	11·1
7	0·00	17·5	0·0	2·2	4·1	17·8
8	—	—	—	2·6	8·1	24·3
12	0·52	15·6	3·3	0·24	19·1	1·0
Childhood (years)						
3	0·94	14·8	5·7	1·2	15·0	7·4
5	0·71	23·8	2·8	1·2	22·8	5·0
Adult (years)						
60	0·00	15·6	0·0	0·00	16·8	0·0

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Cholesterol and cholesterol ester determinations. The changing amounts of cholesterol and its esters in the developing chick cord and whole brain and in the human cord and corpus callosum are shown in Tables 2 and 3. In man the ratio of cholesterol ester to total cholesterol (ester ratio) reached a maximum in the cord before that in the corpus callosum (Fig. 1). In the chick the maximum ester ratio in the cord was reached from about the fourteenth to the sixteenth day of incubation.

Histochemical results

These results are summarized in Table 4. Lipid droplets were seen in variable numbers before the onset of myelination in the chick cord. These droplets were at best only faintly stained by acid haematin while the majority were unstained by OTAN. A few larger droplets, however, were stained greyish-black by the latter method indicating that they contained hydrophobic lipid. Later, in both the chick and human, droplets appeared around the axon: both these droplets and the early myelin sheaths were stained black by OTAN (Figs. 2 and 3). Thus the lipids of

TABLE 3.—ESTER AND FREE CHOLESTEROL LEVELS IN THE DEVELOPING CNS OF THE CHICK

Age (days)	Cord			Whole brain		
	Ester cholesterol (mg/g)	Free cholesterol (mg/g)	% ester	Ester cholesterol (mg/g)	Free cholesterol (mg/g)	% ester
Embryo						
14	1·6	3·4	32·5	—	—	—
16	1·8	3·7	32·3	—	—	—
	2·5	3·4	42·2	—	—	—
18	1·7	7·6	17·8	—	—	—
19	1·1	9·7	10·0	—	—	—
20	0·93	10·6	8·1	—	—	—
21	—	—	—	0·45	6·5	6·4
Chick						
7	—	—	—	0·05	10·2	0·5
9	2·2	21·4	9·3	—	—	—
10	—	—	—	0·00	12·9	0·0
18	1·8	26·2	6·6	—	—	—
29	—	—	—	0·75	10·9	6·4
32	0·00	41·0	0·0	—	—	—
40	0·00	40·0	0·0	—	—	—
43	—	—	—	0·65	10·9	5·7
45	—	—	—	0·00	13·4	0·0
120	—	—	—	0·00	15·4	0·0

the early sheath as well as those of the droplets are hydrophobic. The acid haematin method was faintly positive in the early sheaths while the ferric hydroxamate reaction was negative: this indicated that only small amounts of phospholipid were present. Conversely, in the more mature sheath the staining reactions for phospholipid and

phosphoglyceride were strongly positive and, by now, the sheath was stained red by OTAN indicating the hydrophilic nature of the mature myelin.

Extraction by chloroform-methanol or pyridine, but not by acetone, extinguished the sudanophilia of the lipid droplets. The hydrophobic lipid of the early myelin sheath was dissolved by acetone, a feature in common both to cholesterol esters and triglyceride esters. In the mature sheath extraction by chloroform–methanol

TABLE 4.—HISTOCHEMICAL IDENTIFICATION OF LIPIDS DURING DEVELOPMENT OF THE HUMAN AND CHICK CORD.

Lipid droplets were found in small numbers in the human cord up to the 22nd week of foetal life, early myelin sheaths appeared at this time while mature sheaths were seen from the 30th week onwards. In the chick embryo droplets were seen up to the 16th day of incubation; a few thin myelin sheaths were seen from the 14th day and many mature sheaths were seen at the 20th day.

Method	Lipid droplets	Myelin sheath	
		Early	Mature
ONAB	occ. black	black	red
Acid haematin	nil	slight	blue-black
Ferric hydroxamate	nil	nil	yellow
Nile blue sulphate	nil	nil	nil
Sudan black	black	black	very black

or pyridine failed to remove all the material stained by OTAN, acid haematin or by sudan black. Extraction by acidified solvents, however, extinguished most of this residual staining. This effect of mineral acid could have been due to destruction of the bond between neurokeratin and diphosphoinositide (LE BARON and FOLCH-PI 1956) or by hydrolysis of protein-bound plasmalogens.

Correlation of chromatographic separation with histochemistry

With some extracts of brain and cord successive 10 ml fractions were collected from the alumina column and their sterol content was estimated by the method of HANEL and DAM (1955). The remaining part of each fraction was now divided into half and each was spotted in parallel rows onto Whatman No. 1 paper. One row was treated with the phosphotungstate reagent of DOUSTE-BLAZY *et al.* (1952) while the other half was treated with the Marchi reagent (0·25% osmium tetroxide in an 0·75% aqueous solution of potassium chlorate). In this way the Marchi-positive lipid fractions of the column were identified and were found to be mainly in the cholesterol ester fractions although slight staining was sometimes encountered in the cholesterol zone. Such slight staining was probably due to the decomposition of cholesterol esters on alumina with the liberation of unsaturated fatty acid (KERR

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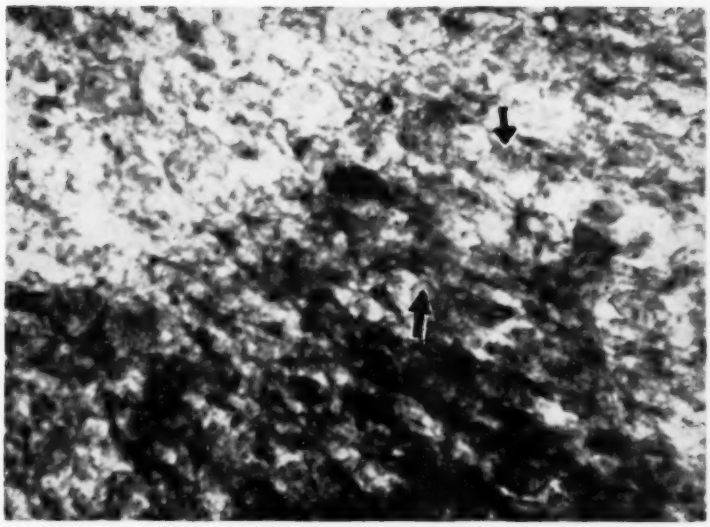

FIG. 2.—Cord of chick embryo after 16 days incubation: positive Marchi reaction in early myelin sheaths (arrows). OTAN, ×900.

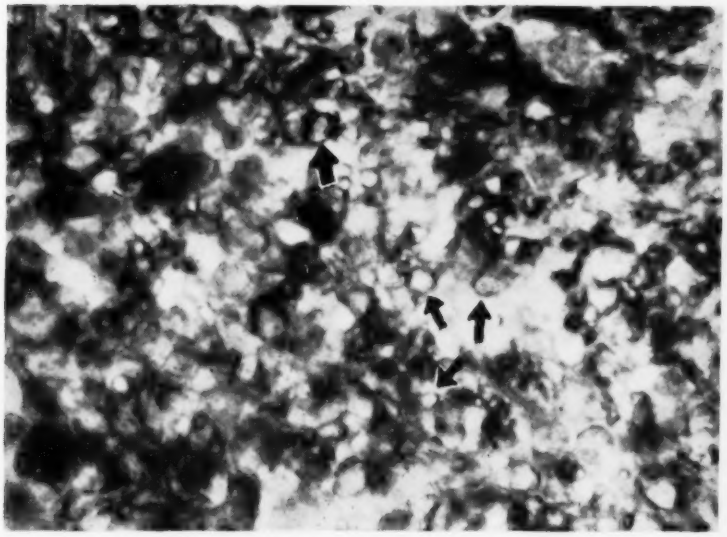

FIG. 3.—Cord of human foetus after 28 weeks gestation: positive Marchi reaction in early myelin sheaths (arrows). Note the droplets in some sheaths (thick arrow). OTAN, ×900.

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and BAULD, 1953). Although the Marchi reagent demonstrates only the unsaturated fatty acids of hydrophobic lipids, there is little doubt that this stain may be used for the general demonstration of cholesterol esters since the proportion of unsaturated acids in these esters is about 85 per cent (KELSEY and LONGENECKER, 1941).

DISCUSSION

This paper describes the identification and estimation of cholesterol esters in the developing central nervous system of the chick and in man. Distinct maxima were found in the ratio of ester to total cholesterol (ester ratio) before birth in the human cord and after birth in that part of the human brain examined. Although cholesterol esters have previously been found in the brain of the embryo chicken (MANDEL, BIETH and STOLL, 1949) their presence in the developing human nervous system has, in the past, been open to doubt. Thus, JOHNSON, MCNABB and ROSSITER (1949) found more than 4 per cent of cholesterol as ester in infant white matter while less than 2 per cent was found in infant grey and adult grey and white matter. Conversely, other workers (BRANTE, 1949; CUMINGS, GOODWIN, WOODWARD and CURZON, 1958) have reported either the complete absence, or the presence of usually small amounts, of esterified cholesterol in human foetal and adult white matter and whole brain. The high ester ratio found by us in the developing human nervous system cannot at first be easily reconciled with the observations of these other workers. These discrepancies could be due to the inherent errors of an analytical method in which esterified cholesterol is determined by the difference between total cholesterol (after alkaline hydrolysis) and free cholesterol (SPERRY and WEBB, 1950). In an attempt to overcome these possible errors our analyses were made in duplicate and neutralized potassium hydroxide was added to the unhydrolysed extract so as to make a more exact control. Furthermore, in order to confirm these analyses and to increase their sensitivity, some extracts were also separated by chromatography on alumina so as to provide a concentrated fraction of ester cholesterol essentially devoid of free cholesterol. The results with these chromatographic fractions always confirmed the figures found by the preliminary chemical analysis of the crude extracts and, moreover, gave direct evidence for the presence of ester. Another, more likely, explanation of the discrepancy between our results and those of other authors may be that they had analysed whole brain or white matter whereas the results presented in this paper refer only to the corpus callosum. Preliminary figures for other parts of cerebral white matter suggest that in these parts the peak in the ester ratio occurs at a different time. Furthermore, multiple analyses of the corpus callosum showed variation within the same area during the ester 'peak'. It follows that a large amount of cholesterol ester in only one region of the brain would remain undetected if this was diluted, in the sample analysed, with large areas of tissue containing no ester at all. This, probably, is the reason why only a small amount of ester was found in the whole-brain samples from the chick.

Histochemical localization of cholesterol esters

The Marchi reaction is widely used to demonstrate histochemical changes in degenerating nervous tissue. WOLMAN (1956, 1957) has suggested that the Marchi reaction depends on the liberation of neutral mucopolysaccharides in the myelin sheath. However, evidence has been presented for an alternative view (ADAMS, 1958,

1959) that unsaturated cholesterol esters are responsible for the staining of degenerating myelin by the Marchi method: solubility studies (WOLFGRAM and ROSE, 1958) and chromatographic separation (ADAMS, to be published) of degenerating myelin also support this view. The present histochemical observations demonstrated the presence of large amounts of hydrophobic lipid in the newly-formed myelin sheath. Since no triglyceride was found in any chromatographic fraction of the CNS this hydrophobic lipid was probably esterified cholesterol.

In the initial Marchi-positive stage of myelination the sheath is only faintly stained by acid haematin. For this reason it is unlikely that this stage corresponds to the phase of myelination in the rabbit brain when phosphosphingoside but little glycosphingoside is present (EDGAR, 1957) for at this time the sheath is stained well by acid haematin (DE ALMEIDA and PEARSE, 1958).

Correlation of the ester cholesterol peak with the onset of myelination

The sudden rise in the ester ratio in the developing nervous system is related to the onset of myelination. Thus, in the human cord the highest ester ratio was found from the 28th to the 38th week of foetal life: this corresponded to the appearance of many new myelin sheaths. BARKER (1902) found that medullation occurred in various tracts of the human cord from the 22nd to the 36th week of foetal life; KEENE and HEWER (1931) record that extensive myelination of the posterior columns starts at the 22nd to the 24th week; although a few myelinated tracts appear in the posterior columns at 24 weeks and more at 28 weeks yet the tractus gracilis was only faintly myelinated at the later date. Likewise, in the chick cord the highest ester ratio was found from the 14th to the 18th day of embryonic life (hatching = 21 days); this approximately corresponds to the most active stage in the appearance of new myelin sheaths in the cord of this species (BENSTED, DOBBING, MORGAN, REID and PAYLING WRIGHT, 1957). In the human corpus callosum the maximum ester ratio was at about the eighth week of extra-uterine life. At this time only a few thin myelin sheaths were seen. This is in accord with LANGWORTHY (1932) who found only light myelination of the corpus callosum in the second month.

It is reasonable to conclude that, in the human cord and corpus callosum, the maxima of esterified cholesterol are coincidental with the initial stages of myelination. It could be argued that the ester was present throughout the brain and was not confined to the myelin sheath. The small amount of ester in grey matter, however, and the histochemical evidence, do not suggest a uniform distribution throughout the brain but rather that it is concentrated in the newly-formed sheath. Although the results presented here indicate that there is a high proportion of ester to free cholesterol in the sheath at the onset of myelination, yet it does not appear that this high ratio is present throughout the maturation of myelin, for this process may not be complete for many months (EDGAR, 1957).

The physiological implication of the peak in the ester ratio is uncertain and this forms a problem for future research. On the one hand, cholesterol esters may be incorporated in the sheath in the initial stages of myelination and then subsequently become diluted by the deposition of large amounts of other lipids so that the esters are only present in trace amounts in the mature myelin sheath. On the other hand, the ester peak may represent an accumulation of a precursor for the formation and deposition of free cholesterol in the maturing myelin sheath.

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SUMMARY

(1) Esterified cholesterol, in large quantity, has been identified and estimated in the developing CNS of the chick and human.

(2) The ratio of ester to free cholesterol reaches a maximum in the human cord about 4 months before the corresponding peak in the corpus callosum.

(3) Hydrophobic lipid, probably esterified cholesterol, has been located by histochemical means in the newly-formed myelin sheath.

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HISTOCHEMICAL INVESTIGATIONS ON SUCCINIC DEHYDROGENASE IN THE CENTRAL NERVOUS SYSTEM—III

ATLAS OF THE MIDBRAIN OF THE GUINEA PIG, INCLUDING PONS AND CEREBELLUM

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THE present article describes in detail the cerebellum and the nuclear pattern of the guinea pig pons and midbrain as defined by a histochemical demonstration of succinic dehydrogenase activity (SDA). The description and accompanying plates continue the author's preceding atlas of the medulla oblongata of the guinea pig (FRIEDE, 1959).

The nomenclature of this atlas corresponds to that of GILLILAN's (1943) description of the midbrain of rat, mouse, and rabbit. Reference is also made to CASTALDI's (1923) terminology for the midbrain of the guinea pig and to the atlas of the rabbit brain by WINKLER and POTTER (1911). Since these prior articles provide a detailed cytoarchitectonic description and mapping of the rodent midbrain, the present article was confined to the chemoarchitectonics of this region.

MATERIAL AND METHODS

The same histochemical tetrazolium technique was employed for the demonstration of SDA as has been outlined in the preceding articles (FRIEDE, 1958, 1959). Young adult guinea pigs were killed by a blow to the neck. The brains were removed from the cranial vault and cut immediately upon sacrifice. Succinic dehydrogenase was demonstrated in frozen sections of unfixed tissue, 60 μ thick, prepared with an improved cutting technique. Both tetrazolium compounds, Tetrazolpurpur (NEUMANN and KOCH, 1953) and Nitro BT (NACHLAS *et. al.*, 1957), were employed. For further details on the author's technique, reference may be made to the earlier reports.

The general appearance of the histochemical reaction and the evaluation of its intensity were considered in the preceding atlas of the medulla oblongata. The reaction with Tetrazolpurpur exhibits dark violet diformazan granules on a diffusely stained orange or yellow background. Nitro BT produces dark violet diformazan granules exclusively without the orange or yellow staining of the background. Nitro BT lowers the threshold of the reaction, thereby demonstrating weaker activity of succinic dehydrogenase in white matter and certain portions of the neuropil. Such differences between the two tetrazolium derivatives, however, do not influence the general pattern of the granular distribution nor the gradation of the reaction between nuclei.

Complete serial sections were made of the entire midbrain of four guinea pigs. Additional random sections were available for comparison as well as material from other experimental investigations with rats and cats. The sections pictured in this article were stained with Tetrazolpurpur. The plates are described in caudo-cranial sequence, directly continuing the atlas of the medulla oblongata.

RESULTS

PLATE I

The section is taken from the pons somewhat cranial to the trigeminal motor nucleus.

The periventricular grey (*gr. cent.*) shows a diffuse arrangement of weak SDA in the neuropil in which a few cell bodies are recognizable by their slightly stronger SDA. The ependyma shows its typically strong SDA, but a narrow subependymal glial layer lacks SDA.

A round area of slightly stronger SDA stands out from the surrounding periventricular grey; this area is identical with the *cell group q* (*q*) of MEESSEN and OLSZEWSKI (1949).

SDA in the *locus coeruleus* (*coe.*) is exceedingly weak. The cytoarchitectonic subdivision of its *pars* α (*coe.* α) of MEESSEN and OLSZEWSKI (1949) shows the least amount in its neuropil. Many of the cell bodies of both portions are recognizable only by their differences in refraction in the otherwise unstained tissue.

The *nucleus radicis mesencephali trigemini* (*N.V. mes.*) exhibits very strong SDA in its characteristic neurons but no SDA in the surrounding tissue.

Mild SDA is found in the neurons and neuropil of the *nucleus marginalis pedunculi cerebelli superior* (*mar. p.c.s.*) or the nucleus parabrachialis of MEESSEN and OLSZEWSKI (1949).

The *nucleus reticularis* of the pons (*ret. p.*) shows strong SDA in its large neurons and their dendrites. The dendrites blend into a reticular neuropil of weak SDA. The medioventral portion of the nucleus exhibits slightly stronger SDA in its neuropil but weaker SDA in its cell bodies.

The *nucleus medianus raphes* (*med. raph.*) of GILLILAN or the *nucleus centralis superior* of MEESSEN and OLSZEWSKI is recognized as a small area of weak SDA on both sides of the *raphe*. The *raphe* itself shows no SDA.

The *nucleus papilioformis* (*ppl.*) of MEESSEN and OLSZEWSKI (1949) forms a distinctly delineated complex by strong SDA in both neurons and neuropil. The texture of the latter is irregular and reticulated.

The *griseum pontis* (*gr. pont.*) shows a diffusely distributed, very strong SDA in both neuropil and cell bodies. On the basis of apparent slight variations in the SDA of portions in the *griseum pontis*, it may be possible to subdivide the nucleus, but more material is required to establish the consistency of these minor differences.

Considerable difficulty was encountered in correlating the cytoarchitectonic and histochemical subdivisions of the nuclei associated with the *lemniscus lateralis*. A large cell group with strong SDA in its neuropil is demonstrated at the ventrolateral surface of the pons; it is identical with the *nucleus caudalis ventralis lemnisci lateralis* (*lem. l. c.v.*) of GILLILAN or the *nucleus lemnisci lateralis ventralis* of MEESSEN and OLSZEWSKI (1949).

PLATE II

The section is determined by the appearance of the caudal portion of the *inferior colliculus*.

The ventral portion of the *periventricular grey* is occupied by the *nucleus dorsalis tegmenti* (*dors. teg.*) and the *nucleus laterodorsalis tegmenti* (*laterodors. teg.*) of CASTALDI. The former is sharply separated from the surrounding grey by strong

SDA in its neuropil in which cell bodies are hardly visible. The *nucleus laterodorsalis tegmenti*, on the other hand, exhibits mild SDA in its neuropil, and cell bodies of stronger SDA are clearly visible. This strong reaction of the cell bodies distinguishes the nucleus clearly from the *central grey* in which equally weak SDA is found in both cell bodies and neuropil. Subdivision of the *latero-dorsal nucleus* as described by GILLILAN is not clearly substantiated by differences in distribution of SDA.

The *area cuneiformis* (*cun.*) appears as an inconspicuous triangular region with weak to mild SDA in its neuropil. A few neurons are visible by their stronger SDA. Thus the distribution of SDA in the *area cuneiformis* closely resembles that in the *periventricular grey*. The *area cuneiformis* appears sharply delineated laterally toward the *colliculus inferior* (*coll. inf.*) but medially it blends into the *nucleus laterodorsalis tegmenti*.

The *nucleus radicis mesencephali trigemini* (*N. V. mes.*) crosses the *area cuneiformis*.

The *nucleus medianus profundus* (*med. prof.*) or *nucleus ventralis tegmenti* of GUDDEN is separated from the *nucleus dorsalis tegmenti* by the fiber bundles of the *fasciculus longitudinalis medialis* (*f.l.m.*). The *nucleus medialis profundus* exhibits mild SDA in its neuropil but strong SDA in its star shaped neurons.

The *nucleus dorsalis raphes* (*dors. raph.*) and the annular nucleus of the *fasciculus longitudinalis medialis* are not readily discernable, since both exhibit diffusely arranged, weak SDA as found in the *central grey*.

The distribution of SDA in the *nucleus medianus raphes* (*med. raph.*) and the *griseum pontis* (*gr. pont.*) was described with Plate I.

In this section a circumscribed cell group of mild SDA appears within the *nucleus marginalis pedunculi cerebelli superior* (*mar. p.c.s.*). It is not quite clear whether or not this cell group reflects displaced portions of the *red nucleus* as described by GILLILAN (1943).

The *nucleus caudalis ventralis lemnici lateralis* (*lem. l.c.v.*) of GILLILAN shows the same intensity of SDA as in Plate I, but its neuropil appears perforated by fibre bundles. Two subdivisions of this nucleus were recognized by MEESSEN and OLSZEWSKI (1949): the *nucleus lemnisci lateralis ventralis* and the *nucleus lemnisci lateralis dorsalis* (Plate XIV of these authors). The ventral subdivision appears in Plate I of this atlas and the dorsal subdivision in Plate II. GILLILAN did not consider the two portions distinct and included both under the name *nucleus caudalis ventralis lemnisci lateralis*. They seem clearly discernible, however, in a representative photomicrograph of GILLILAN's report (Plate 7 of this author).

The *nucleus dorsalis lemnisci lateralis* of GILLILAN appears indiscernible from the *area cuneiformis*.

PLATE III

The section shows the *mesencephalic tectum* at the rostral extent of the *inferior colliculus*. A tangential section of the *superior colliculus* appears above the *inferior colliculus*.

The medioventral portion of the *colliculus inferior* (*coll. inf.*), particularly in its caudal part, exhibits strong SDA in the neuropil with no neurons visible. Its delineation from the adjacent tissue is sharp. The dorsolateral portion of the *colliculus inferior*, particularly the oral part of the nucleus, however, shows little SDA in the somewhat scattered neuropil but strong SDA in the cell bodies. This type of nuclear subdivision is prominent throughout the brain.

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PLATE II
ef. cer.
dors. lat.
laterodors. leg.
N. V. mes.
cun.
ol. inf.
f. l. m.
dors. raph.
med. raph.
med. prof.
ref. p.
p. c. s.
mar. p. c. s.
lem. l. c. v.
ef. post.
pyr.

PLATE I
ef. cer.
q
f. l. m.
med. raph.
ref. p.
cue.
cue. a.
N. V. mes.
p. c. s.
mar. p. c. s.
lem. l. c. s.
ppl.
pyr.
ef. post.
p. c. m.

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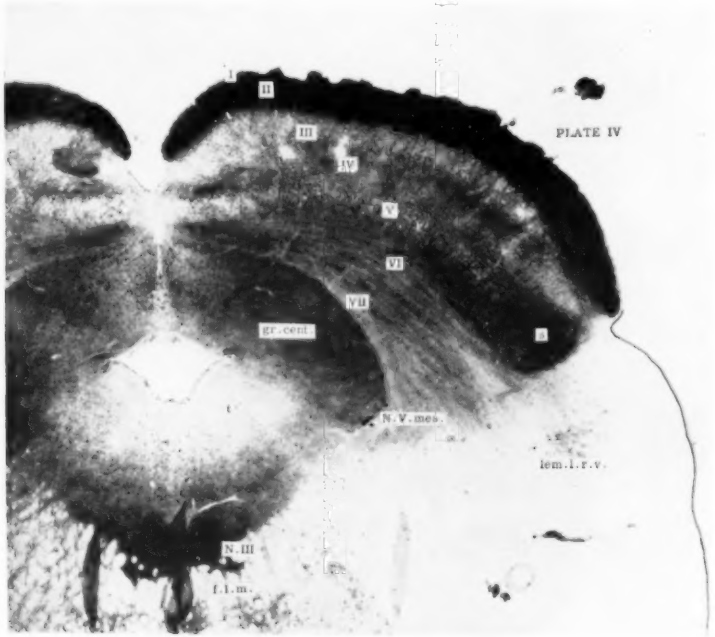

PLATE IV

PLATE III

The neuropil of the *central grey* (*gr. cent.*) displays a mild, diffusely arranged SDA, cell bodies being barely visible by their more intense staining. Subdivision of the *central grey* into a mediodorsal, laterodorsal and a ventral nucleus, as done by CASTALDI (1923), is not supported by a differential distribution of SDA. Dorsal to the *nucleus laterodorsalis tegmenti* (*laterodors. teg.*), however, an area (*t*) of no or exceedingly weak SDA is seen in the *central grey*. This area considerably enlarges more rostrally. The *nucleus dorsalis tegmenti* (*dors. teg.*) and the *nucleus laterodorsalis tegmenti* were described with Plate II. Bits of the *area cuneiformis* (*cun.*) still present at this level, show SDA similar to that of the *central grey*.

The *nucleus centralis lateralis* does not happen to appear in any of the Plates; the distribution of SDA in it, though, is similar to that in the *nucleus laterodorsalis tegmenti*.

The *nucleus dorsalis raphes* (*dors. raph.*) is larger than in Plate II. Its diffusely arranged, weak SDA seems to extend the *central grey* ventrally. The various portions or branches by which the nucleus extends between adjacent nuclei (GILLILAN) are not distinguished by SDA.

The *nucleus dorsalis lemnisci lateralis* again is not discernible from the *area cuneiformis*. The *nucleus caudalis ventralis lemnisci lateralis* (*lem.l.c.v.*) shows strong SDA as in Plate II, but although the reaction is somewhat weaker in the *nucleus rostralis ventralis lemnisci lateralis*, a definite boundary between the two nuclei could not be discerned with certainty.

PLATE IV

The section is taken through the caudal portion of the *superior colliculus* and demonstrates its laminar structure.

The *stratum griseum superficiale* (II) shows very strong, diffusely arranged SDA of the neuropil without recognizable cell bodies. Less SDA is present in the superficial *stratum zonale* (I). The *stratum medullare superficiale* (III) exhibits weak SDA in a reticular neuropil situated between fibre bundles. A lamina of small neurons with strong SDA is found in the deeper part of layer III. These cells are also found in the upper part of the *stratum griseum intermedium* (IV). The SDA in layer IV is otherwise mild and diffusely distributed in a reticular neuropil. The *stratum medullae intermedium* (V) is demarcated by its large fiber bundles without SDA. The neuropil of the *stratum griseum profundum* (VI) exhibits mild, diffusely arranged SDA, and a few cell bodies are recognizable through their somewhat deeper staining. The IVth and VIth layers join laterally to form a distinct large complex (*s*) which contains diffusely arranged, mild SDA in the neuropil. This complex more rostrally appears separated as a distinct nucleus (Plates VI and VII). The *stratum medullare* profundum (VII) borders the *central grey* by its fibre bundles. The scattered cells of the *nucleus radicis mesencephali trigemini* (*N.V. mes.*) are seen at this borderline.

The neuropil of the *central grey* (*gr. cent.*) shows its typically weak SDA. The ventromedial portion of exceedingly weak SDA (*t*, Plate III) has considerably enlarged at this level and within it some small neurons with mild SDA are found. The dorsal portion of the *central grey* is occupied by a group of cell bodies with stronger SDA than that of the neuropil.

The *ependyma* covering a small protrusion of the *raphe* dorsally just caudal to the *subcommissural organ* completely lacks SDA.

The most rostral extension of the *nucleus rostralis ventralis lemnisci lateralis* (*lem.l.r.v.*) is seen at the lateral margin of the *tegmentum*. It is characterized by a few scattered neurons with mild SDA.

The *nucleus trochlearis* and the *nucleus oculomotorius* (*N.* III) are discernible only by their localization and not by differences in staining. The appearance of the *oculomotor nucleus* in this Plate is obscured by folds in the section. Such folds are a major obstacle in the preparation of large sections, since fixation of a thick section induces unlike expansion of grey and white matter.

PLATE V

The level of this section is identified by the magnocellular portion of the *red nucleus* and by the *interpeduncular nucleus*.

The appearance of the *colliculus superior* (*coll. sup.*) and of the *central grey* (*gr. cent.*) is the same as described with Plate IV.

Owing to the reaction of the neuropil, the *nucleus Darkschewitsch* (*Dark.*) in the ventral portion of the *central grey* appears as an area of strong SDA in which neurons are visible by their slightly stronger SDA. The adjacent *nucleus interstitialis of Cajal* (*interstit.*) is less conspicuous because of the weaker SDA in its reticular neuropil, but small neurons of strong SDA are present.

The *nucleus oculomotorius* (*N.* III) exhibits very strong SDA in both neurons and neuropil similar to motor nuclei of the medulla oblongata. The *caudal Edinger Westphal nucleus* (*Ed. West.*) stands in marked contrast by its almost complete lack of SDA in both neurons and neuropil.

The *nucleus linearis of Castaldi* (*lin.*) is very difficult to recognize because of the weak SDA in its scattered small neurons and the absence of SDA in its neuropil. The two inconstant, small areas of strong SDA demonstrated in this Plate near the midline are probably dislocated portions of the *oculomotor nucleus*.

The *pars magnocellularis* of the *nucleus ruber* (*rub. mag.*) exhibits very strong SDA in its large neurons. The scattered neuropil between these neurons shows considerably less SDA than is found in the neuropil of motor nuclei of cranial nerves.

The grey associated with the *ansulate commissure of Gillilan* is probably identical with an area adjacent to the *red nucleus* with weak SDA in the neuropil and scattered cell bodies; however, in some of the nuclei of this medioventral region, such as the *nucleus of the decussation of Forel*, the SDA is weak and inconspicuous to a degree which obviates distinction of nuclei.

The *nucleus interpeduncularis* (*interped.*) shows exceedingly strong SDA diffusely distributed in the neuropil of its ventral portion. The dorsal portion of the nucleus exhibits an unique pattern with crossing layers of neuropil. No cell bodies are recognizable except for some minute cells with strong SDA at the dorsal circumference of the nucleus. Vascular spaces appear as large, optically empty tissue lacunae. Only a few nuclei of the central nervous system exhibit as strong SDA as does the *interpeduncular nucleus*.

The *pars reticularis* of the *nucleus niger* (*nig. ret.*) shows scattered neurons of mild SDA with long dendrites forming a loose network which blends into a neuropil of slightly less SDA. The interval between the *pars reticularis* and the *pars compacta* (nig. comp.) is not sharp. The *pars compacta* may easily be overlooked because of exceedingly low SDA in both its neurons and neuropil. Only by differences in light

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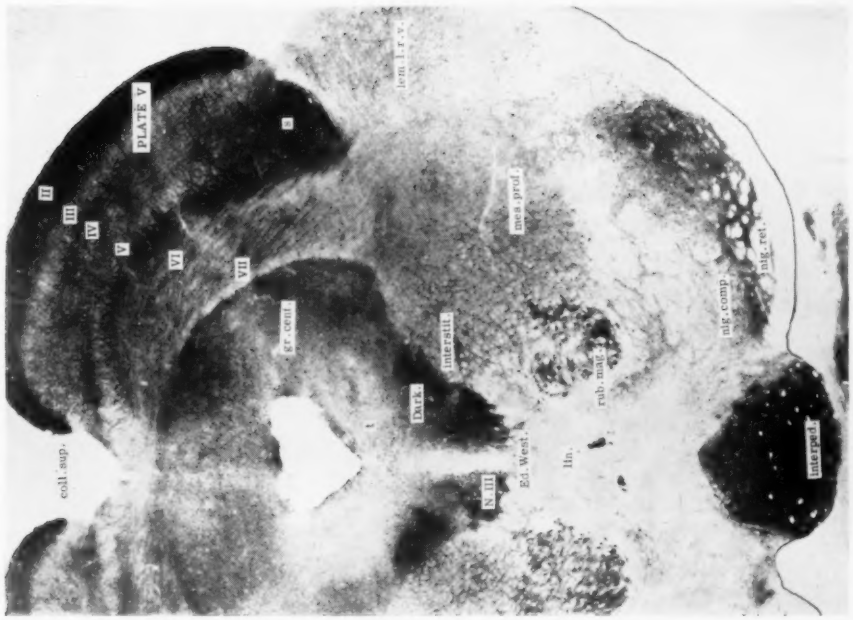
PLATE V
I
II
III
IV
V
VI
VII
a
coll. sup.
gr. cent.
Dark.
interstit.
N. III
Ed. West.
IIn.
mes. prol.
rub. mag.
lem. l. r. v.
nig. comp.
nig. ret.
interpd.

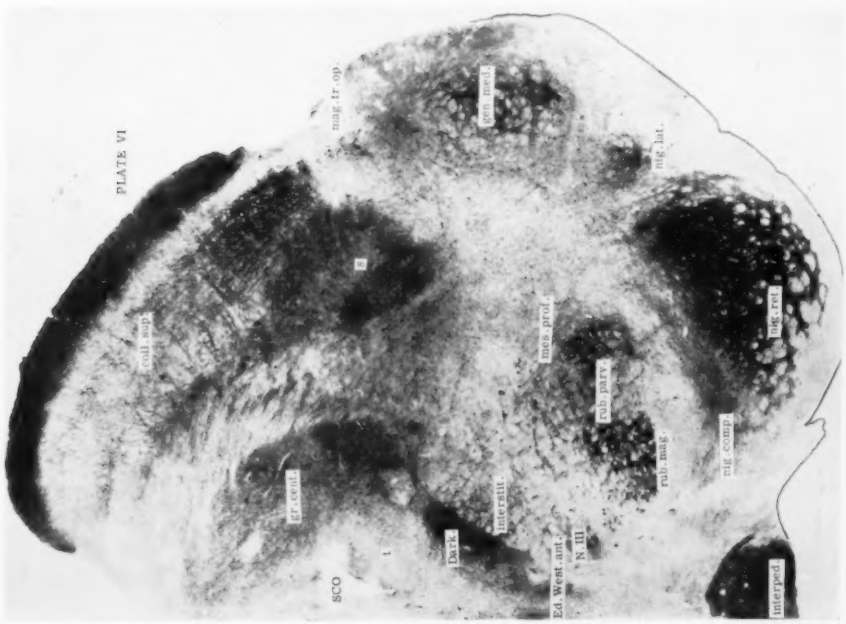
PLATE VI
coll. sup.
gr. cent.
Dark.
interstit.
Ed. West. ant.
N. III
SCO
a
mag. tr. op.
com. med.
mes. prol.
rub. pars.
rub. mag.
nig. lat.
nig. ret.
nig. comp.
interpd.

PLATE VII

coll. sup.
n. com. post. ac.
n. com. post. p.
n. com. post. a.
gr. cent.
mag. comp. p.
SCO
Dark
interstit.
Ed. West. amb.
N. III
post. thal.
mes. prod.
rub. part.
rub. mag.
r. opt. bas.
nig. comp.
nig. lat.
nig. ret.
interped.

refraction are the neurons discernible from a background of unstained or but slightly stained tissue.

The *nucleus mesencephalicus profundus* (*mes. prof.*) will be described with Plate VI.

A few neurons with mild SDA are found at the lateral margin of the section and constitute the rostral pole of the nucleus *rostralis ventralis lemnisci lateralis* (*lem.l.r.v.*).

PLATE VI

The level of the section is indicated by the cranial pole of the oculomotor nucleus and the caudal extent of the *medial geniculate body*.

The laminar texture of the *colliculus superior* (*coll. sup.*) is the same as that described with Plate IV, but the lateral complex (*s*) of the IVth and VIth layers is enlarged to form a distinct nucleus. That this enlargement is not the *posterior thalamic* nucleus is demonstrated by Plate VII.

Some scattered neurons with strong SDA between the fibres of the optic root characterize the *nucleus magnocellularis tracti optici* (*mag. tr. opt.*).

The *central grey* (*gr. cent.*) was described with Plates IV and V. The *nucleus of Darkschewitsch* (*Dark.*) and the *nucleus interstitialis of Cajal* (*interstit.*) are approximated more closely but are discernible by the typical distribution of SDA in them as described with Plate V. The dorsal pole of the *nucleus oculomotorius* (*N. III*) shows as an area of mild SDA.

The rostral portion of the nucleus of Edinger and Westphal or the *nucleus medianus anterior* (*Ed. West. ant.*) shows very weak SDA in its neuropil and cell bodies. The SDA in this nucleus, however, appears slightly stronger than that in the *caudal Edinger Westphal nucleus*.

The transition between the *pars magnocellularis* and the *pars parvocellularis of the nucleus ruber* (*rub. parv.; rub. mag.*) is characterized by a slight increase of SDA in the neuropil.

The region between the *red nucleus* and the *medial geniculate body* is occupied by the *nucleus mesencephalicus profundus* (*mes. prof.*) with mild SDA in both neuropil and cell bodies. A comparison of the appearance of this region in Plates V, VI, and VII shows that there is no clear borderline between the *nucleus mesencephalicus profundus*, the *nucleus interstitialis* of *Cajal*, and the parvocellular portion of the *red nucleus*. Such evident transition parallels the cytological similarities of these nuclei (GILLILAN, 1943; STENGEL, 1924).

The subdivisions of the *nucleus niger*, *pars reticularis* (*nig. ret.*), *pars compacta* (*nig. comp.*), and *pars lateralis* (*nig. lat.*), are easily seen. SDA is distributed similarly in the *pars lateralis* and the *pars reticularis*.

The *nucleus interpeduncularis* (*interped.*) was described with Plate V. The *nucleus geniculatus medialis* (*gen. med.*) will be described with Plate VII.

PLATE VII

The level of the section is indicated by the appearance of the posterior commissure.

The laminar architectonics of the *colliculus superior* (*coll. sup.*) are disturbed through its blending into the *nucleus pretectalis*. In place of discernible tectal lamina, three areas appear which are difficult to correlate with the known cytoarchitectonic subdivisions of this region. A portion with strong SDA in both neuropil

and neurons (*u*) is found laterally; it probably includes neurons of the magnocellular nucleus of the optic tract. An elongated area of diffuse SDA in the neuropil but without recognizable cell bodies (*v*) is found below the superficial layer of the *superior colliculus*. A third area (*w*) exhibits strong SDA in its small neurons but lacks SDA in its neuropil. The lateral enlargement of the IVth and VIth tectal lamina (*s*) is still visible but reduced in size. At this level its becomes clearly discernible from the caudal pole of the *nucleus posterius thalami* (*post. thal.*) which appears as a circumscribed area with mild, diffuse SDA in its neuropil.

The nucleus *interstitialis commissurae posterioris* (*n. com. post.*) shows mild SDA in its neurons and weak SDA in its neuropil throughout—in the *pars supracommissuralis* (α), *pars intracommissuralis* (β), and *pars infracommissuralis* (γ). The *nucleus interstitialis magnocellularis commissurae posterioris* (*mag. com. p.*), however, is discernible through strong SDA in its larger cell bodies situated between diverging fibre bundles.

The *nucleus of Darkschewitsch* (*Dark.*) and the *nucleus interstitialis of Cajal* (interstit.), though forming one complex, may be distinguished easily by their different histochemical texture as described with Plate V. The *nucleus medianus anterior* (*med. ant.*), *nucleus ruber* (*rub.*), *nucleus mesencephalicus profundus* (*mes. prof.*), *nucleus niger* (*nig. comp., nig. ret, nig. lat.*), *magnocellular nucleus of the optic tract* (*mag. tr. opt.*), and the *central grey* (*gr. cent.*) were described with Plates V and VI. Small bits of the *nucleus interpeduncularis* (*interped.*) appear with their typically very strong SDA.

The *nucleus of the basal optic root* (*r. opt. bas.*) shows an accumulation of medium sized cells with strong SDA in a scattered neuropil of mild SDA. Stronger SDA clearly separates this nucleus from the *pars compacta* of the nucleus niger. This division is not so clear with conventional staining techniques (GILLILAN, 1943).

Part of the *nucleus geniculatus medialis* (*gen. med.*) is included in the section. As is so frequently found in the brain's nuclei, the medial geniculate nucleus is subdivided into a lateral portion with strong SDA in the neuropil but without recognizable cell bodies and a medial portion with strong SDA in the cell bodies but weak in the neuropil. In addition, a dorsal area of mild SDA in the neuropil is discernible.

CEREBELLUM

The *dentate nucleus* appears as a homogenously stained band with strong SDA in both its cell bodies and neuropil. The intensity and distribution of SDA in this nucleus closely resemble those in the inferior and superior *olivary nuclei*. The *nuclei fastigii, globosus*, and *emboliformis* exhibit somewhat weaker SDA in their neuropil and are not clearly demarcated by differences in either intensity or distribution.

The molecular layer of the cerebellar cortex exhibits strong, diffusely distributed SDA. The thicker dendrites of the Purkinje cells, which are slightly more strongly stained, may be seen in the deeper portions of the layer. The bodies of the Purkinje cells stand out through their very strong SDA, but the intervals separating them, which are loaded with glial cells, show no SDA. In the granular layer somewhat stronger SDA is found with a flocculated distribution of very intensely stained small clusters.*

The distribution of cytochrome oxidase in the cerebellar cortex, excellently

* The relative intensity of SDA in the cerebellar cortex may be adjudged by consulting the plates of the author's earlier atlas of the medulla oblongata.

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demonstrated by CAMPBELL (1939), is remarkably similar to the distribution of SDA. CAMPBELL attributed the flocculated appearance of the granular layer to grouping of the granular cells. On isolated tissue elements, it has been proved, however, that the strong SDA of the granular layer is located in the synaptic *glomerula cerebellaria* and that almost no SDA is found in the granular cell bodies (FRIEDE, 1959). The enzyme activity of the *glomerula cerebellaria*, therefore, is solely responsible for the flocculated appearance of the granular layer. This observation seems important in interpretation of the results obtained with microchemical tissue samplings; values from samples from the molecular layer may be attributed to dendrites, but values from samples from the granular layer do not reflect necessarily its extreme density of cell bodies. The SDA content of the *glomerula cerebellaria* is paralleled by their richness in mitochondria (ORTIC-PICON and PEREZ-LISTA, 1929).

As judged from the many illustrations of the distribution of SDA in the cerebellar cortex which already have been published (BOURNE and MALATY, 1953; SHIMIZU, MORIKAWA and ISHI, 1957; SHIMIZU and MORIKAWA, 1957; FRIEDE, 1957; NACHLAS *et al.*, 1957) and the authors own investigations, the distribution of SDA in the cerebellar cortex of various mammals including man seems identical. Minor differences in distribution, however, may occur between species; e.g. in studying a series of Sprague–Dawley rats, a very large number of Purkinje cells with mild to weak SDA was found, and the *glomerula cerebellaria* were individually discernible owing to weak SDA in intervening cells.

DISCUSSION

Particular attention was paid to the appearance of the SDA reaction and its relationship to cell bodies, dendrites, and neuropil in the discussion of the author's preceding atlas of the medulla oblongata. The relationship of SDA to cell statistics will be considered in a forthcoming section of the atlas. For the present, a detailed discussion of the relationship of SDA to vascularization of tissue is provided. Where the distribution of SDA is considered in areas of the brain not yet described in this series, reference may be made to the work of SHIMIZU and MORIKAWA (1957).

Extensive measurements of capillarization have been made in the rat (CRAIGIE, 1920, 1921, 1945) in which the distribution of SDA is known to be similar to that in the guinea pig. Changes in the vascularization of parts of the guinea pig's brain have been studied by PETREN (1938). Other authors, as cited subsequently, have studied the capillarization of the brains of cats, rabbits, monkeys and man. Their data seem, nevertheless, comparable to the SDA distribution in the guinea pig, for both the pattern of vascularization and the pattern of distribution of SDA are fairly constant throughout various species of mammals (CRAIGIE, 1945).

The distribution of SDA in the cerebellar cortex is paralleled by its pattern of capillarization (OEGG, 1857; ABY, 1899; OBERSTEINER, 1912; FAZZARI, 1924; PFEIFFER, 1930; *et al.*). The capillarization indices* for the cat are 2·0 for the molecular layer and 2·5 for the granular layer (CAMPBELL, 1939). This pattern of vascularization in turn is paralleled by the distribution of mitochondria (SCHARRER, 1945). The interpeduncular nucleus which exhibits about the strongest SDA in the midbrain is rich in both capillaries and mitochondria (SCHARRER, 1945).

* Capillarization indices are computed as multiple values for the hippocampal *lamina radiata*.

The very strong SDA in the superior and inferior *olivary nuclei*, the *dentate nucleus*, and the medial *vestibular nucleus* is reflected by their dense capillarization (rat, CRAIGIE, 1920); however, the differences in the capillarization of motor and sensory nuclei (CRAIGIE, 1920) are not as clearly reflected by differences in SDA. The predominance of SDA in the cell bodies of the motor nuclei and in the neuropil of the sensory nuclei may account for this lack of proportionality.

The strong SDA in the neuropil of the *putamen* and weak SDA in the neuropil of the *pallidum* (SHIMIZU and MORIKAWA, 1957) are reflected in capillary indices of 1·8 and 1·1 respectively (cat, CAMPBELL, 1937, 1939). In the thalamus, SDA is very strong in the *lateral geniculate body* and the anterior and ventral *thalamic nuclei* and is weak in the lateral and medial *thalamic nuclei* (SHIMIZU and MORIKAWA, 1957). This pattern is reflected by vascularization indices of 2·2–3·1 for the lateral *geniculate body*, 2·2 for the anterior *thalamic nucleus*, 2·0 for the ventral *thalamic nucleus*, and 1·7 for the *dorsomedial nucleus* (cat, CAMPBELL, 1939).

In the cerebral cortex SDA is strongest in the third and fourth layers and grades off in the fifth and sixth layers. Distribution of SDA in the cerebral cortex is paralleled by the vascularization of the cortical laminae: Lamina I, 2·0; II, 2·1; III, 2·4; IV, 2·5; V, 2·1; VI, 2·0 (parietal cortex, cat, CAMPBELL, 1939, also rat, CRAIGIE, 1921; rabbit and monkey, LORENTE DE NO, 1927; cat, TALBOTT, WOLFF and COBB, 1929; cat and man, PFEIFFER, 1930; and man, OBERSTEINER, 1912).

The definitely lower capillarization of the *hippocampus* (cat, CAMPBELL, 1939; rabbit, COBB, 1929; man, PFEIFFER, 1930) is reflected by weaker SDA. Even minor differences in vascularization seem to be paralleled by differences in SDA. The *stria* of *Gennari* in the visual cortex is not distinguished by a particular capillarization (PFEIFFER, 1930) and also is not distinguished by a particular distribution of SDA.

In the *glomerula olfactoria* (PFEIFFER, 1930; SCHARRER, 1945) prominent capillarizations are reflected by strong SDA (ORTMANN, 1957).

The intimate biochemical relationship of SDA and cytochrome oxidase was discussed in the preceding section of the atlas. Both enzymes are closely related in the mitochondria (HOGEBOOM *et al.*, 1946). Accordingly, in the brain the distribution of cytochrome oxidase (ROIZIN, 1955) is almost identical with the distribution of SDA (SHIMIZU, MORIKAWA and ISHI, 1957). CAMPBELL's results with the indophenol reaction in the *putamen*, *pallidum*, and the *cerebral* and *cerebellar cortices* are remarkably similar to the author's result with the tetrazolium technique. CAMPBELL's data on the vascularization of the *thalamus* are better reflected by the tetrazolium technique than by the indophenol reaction. A general positive correlation between the indophenol reaction and the values obtained by capillary measurements exists (CAMPBELL, 1939; MEATH and POPE, 1950). Because of the association of SDA and cytochrome oxidase in the mitochondria, the extent of vascularization and the richness of mitochondria (SCHARRER, 1945), as well as the amount of oxygen consumption (DUNNING and WOLFF, 1936, 1937), are probably also correlated.

The strong enzyme activity in the neuropil of most nuclei parallels their dense capillarization (SCHARRER, 1937, 1945; DUNNING and WOLFF, 1936, 1937; DRUMMOND, 1944). No known relation exists between the enzyme activity in neuropil and in cell bodies. It may be strong in both, or weak in one and strong in the other. The sum of the metabolic requirements of both neuropil and cell bodies, therefore, will determine the extent of vascularization (SCHARRER, 1944); SCHARRER (1939) has

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ably demonstrated this to be true for the *cerebellar granular cortex* of the opossum. Only if similar enzyme activity were present in both the neuropil and the cell bodies, would the cell density reflect the degree of vascularization.

Functional changes seem to accompany changes of both SDA and vascularization. The postnatal increase of SDA in the nuclei of the rat's brain, as described in the first article of this series (FRIEDE, 1959*a*), is accompanied by an increase in vascularization (CRAIGIE, 1924). The motor nuclei double in capillarization between the fifth and the twentieth days, and some sensory nuclei and the superior and inferior *olivary nuclei* triple in vascularization during the same period (computed from Craigie's data). This difference may account for the late increase of SDA in the latter nuclei. Moreover, the very delayed development of the permanent pattern of SDA in the *cerebellum* is paralleled by its delayed capillarization. CRAIGIE's findings have been supported by studies on the development of capillarization in the human *cerebellum* (NIEMNIEVA, 1950; NIEMNIEVA and TERVILÄ, 1953) and the motor nuclei of the cranial nerves (MALECI, 1944).

Changes in the diameter and blood content of capillaries in the brain were described following exposure of rats to simulated altitude (MERCKER and SCHNEIDER, 1949). Succinoxidase in brain tissue was found increased under similar conditions (TAPPAN *et al.*, 1956).

The vulnerability of tissue generally increases with its metabolic activity. Damage resulting from a complete arrest of cerebral circulation (WEINBERGER, GIBBON and GIBBON, 1940, and many other investigators) seems to occur predominantly in regions of strong SDA; however, since so many other factors are involved in the development of damage (HICKS, 1950), it is not possible at this time to predict the vulnerability of a nucleus from its normal enzyme content. A causal relationship, nevertheless, may exist between the long survival time of the vagal centre following complete circulatory arrest (HEYMANS *et al.*, 1934, 1938; KALLE, 1933) and the very weak SDA in the dorsal *vagal nucleus*. Data on the vascularization of the dorsal *vagal nucleus*, as well as of some other nuclei with weak SDA, are not found in the literature.

Some nuclei with weak SDA, though, clearly show no positive relationship between vascularization and SDA. The vascularization of the *substantia nigra*, considered as a whole, is low compared with that of the *oculomotor nucleus*, the *red nucleus* or the *subthalamic nucleus*, but its *pars compacta* contains a considerably denser capillary network than does its *pars reticularis* (FINLEY, 1936). SDA, however, is distributed inversely, being nearly absent in the *pars compacta* and mild in the *pars reticularis*.

In nuclei in which SDA does not follow vascularization, the basic metabolism may not predominantely involve the Krebs cycle. The *supraoptic* and *paraventricular nuclei*, for example, show the richest capillarization among all the brain's nuclei (FINLEY, 1938; CRAIGIE, 1940) but rather weak SDA (SHIMIZU and MORIKAWA, 1957). This rich vascularization does not reflect a high demand for oxygen, since the nuclei are quite resistant to an arrest of circulation (GRENELL and KABAT, 1947). The particular neurosecretory function of these nuclei probably accounts for their outstanding vascularization rather than a high oxidative metabolism.

The poor correlation of vascularization and SDA in the *substantia nigra*, therefore, may reflect an exceptional metabolic behavior of this nucleus. Its content of melanin may be imputed. Another possibility is suggested by the differences in the distribution

of succinic dehydrogenase, diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase found in the nephron (FARBER *et. al.*, 1956) and in the hepatic lobule (SCHUMACHER, 1957). Such differences in the distribution of oxidative enzymes may account for disproportionalities between SDA and vascularization. Thus, investigations on the distribution of the above enzymes in the brain have been started.

The most striking discrepancy between SDA and vascularization exists in the *nucleus coeruleus*. This nucleus almost completely lacks SDA but ranks second in capillarization among all the brain's nuclei—about equalled by the paraventricular nucleus and surpassed only by the *supraoptic nucleus* (FINLEY and COBB, 1940). Experimental evidence accumulated for a pneumotactic centre in the *nucleus coeruleus* (TANY and RUCH, 1951; JOHNSON and RUSSELL, 1952; BAXTER and OLSZEWSKI, 1955). That this nucleus has low SDA agrees well with other reports of an extended survival time of the respiratory centre (30 min after complete arrest of circulation as observed by HEYMANS *et. al.*, 1934, 1938) and of the support of prolonged gasping by anaerobic glycolysis (SELLE, 1944; HIESTADT *et al.*, 1944, 1945; HIMWICH *et. al.*, 1941, 1942). The rapidly diminishing resistance of the respiratory centre to asphyxia after birth (KABAT, 1940; SELLE and WITTEN, 1941) may be related to the outstanding increase of SDA in the *nucleus coeruleus* after birth (FRIEDE, 1959).

On the other hand, the most widely accepted site of the respiratory center in the lower medulla oblongata (FINLEY, 1931; GESELL *et. al.*, 1936; PITTS, MAGOUN and RANSON, 1939; BAKER *et. al.*, 1950) generally exhibits no exceptional enzyme distribution. Cell groups of the reticular formation near the raphe, defined by stronger SDA of their neuropil, about coincide with the region of the respiratory centre described by PITTS, MAGOUN and RANSON (1939), but not clearly with the site of either expiratory or inspiratory areas. The individual variability of these cell groups does not ease interpretation; their existence in cats and dogs has been established.

The striking histochemical differences of the centres supposedly involved in control of respiration may explain the virtually opposed statements expressed in the literature for vulnerability or survival time of the respiratory centers.

SUMMARY

The detailed neuroanatomical distribution of succinic dehydrogenase activity in the midbrain of the guinea pig is described and demonstrated in Plates following the format of an atlas. A short description of the distribution of enzyme activity in the cerebellum is provided. Particular attention is paid to the gradation of the reaction among nuclei and to the distribution of the reaction in cell bodies and neuropil. In the discussion, differences in the vascularization of nuclei are compared with the distribution of succinic dehydrogenase activity. There is a good agreement between the distribution of enzyme activity and vascularization throughout the brain, but a few nuclei exhibit a clearly exceptional behavior.

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INDEX OF ABBREVIATIONS AND SYNONYMS

If not stated otherwise, the terminology refers to GILLILAN (1943); the tectal layers are designated according to WINKLER and POTTER (1911).

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coe.:	Nucleus coeruleus
coe. α:	Nucleus coeruleus, pars α (MEESSEN and OLSZEWSKI, 1949)
coll. inf.:	Colliculus inferior
I:	Stratum zonale
II:	Stratum griseum superficiale
III:	Stratum medullare superficiale
IV:	Stratum griseum intermedium
V:	Stratum medullare intermedium
VI:	Stratum griseum profundum
VII:	Stratum medullare profundum
cun.:	Area cuneiformis
Dark.:	Nucleus of Darkschewitsch
dors. raph.:	Nucleus dorsalis raphes
dors. teg.:	Nucleus dorsalis tegmenti
Ed. West.:	Caudal Edinger Westphal nucleus, main nucleus of Edinger and Westphal
Ed. West. ant.:	Nucleus medianus anterior (nucleus of Edinger and Westphal, Pars rostralis)
f.l.m.:	Fasciculus longitudinalis medialis
gen. med.:	Nucleus geniculatus medialis
gr. cent.:	Periventricular or central grey (griseum centrale, Hoehlengrau, HG in the preceding atlas of the medulla oblongata)
gr. pont.:	Griseum pontis (nuclei pontis)
interped.:	Nucleus interpeduncularis (ganglion interpedunculare)
interstit.:	Nucleus interstitialis of Cajal (nucleus interstitialis fasciculi Longitudinalis medialis)
n. com. post.:	Nucleus commissurae posterioris
α:	Pars supracommissuralis
β:	Pars intracommissuralis
γ:	Pars infracommissuralis
laterodors. teg.:	Nucleus laterodorsalis tegmenti (Castaldi)
lin.:	Nucleus linearis (Castaldi)
N. III:	Nucleus nervi oculomotorii
N. V. *mes.:*	Nucleus radicis mesencephali trigemini
lem. l.c.v.:	Nucleus caudalis ventralis lemnisci lateralis
lem. l.r.v.:	Nucleus rostralis ventralis lemnisci lateralis
l. l.:	Lemniscus lateralis
nig. ret.:	Nucleus niger, zona reticulata
nig. comp.:	Nucleus niger, zona compacta
nig. lat.:	Nucleus niger, pars lateralis
mag. com. p.:	Nucleus interstitialis magnocellularis commissurae posterioris
mag. tr. opt.:	Large celled nucleus of the optic tract (nucleus lenticularis Mesencephali)
mar. p.c.s.:	Nucleus marginalis pedunculi cerebelli superioris (nucleus Parabrachialis)
med. prof.:	Nucleus medialis profundus (nucleus rostralis tegmenti of Gudden)

mes. prof.:	Nucleus mesencephalicus profundus (nucleus lateralis profundus Mesencephali)
med. raph.:	Nucleus medianus raphes (nucleus centralis superior of MEESSEN and OLSZEWSKI, 1949)
post. thal.:	Nucleus posterius thalami
p.c.s.:	Pedunculus cerebelli superior
p.c.m.:	Pedunculus cerebelli medius (brachium pontis)
ppl.:	Nucleus papilioformis (MEESSEN and OLSZEWSKI, 1949)
pyr.:	Tractus pyramidalis
q:	Cell group q of MEESSEN and OLSZEWSKI (1949)
r. opt. bas.:	Nucleus of the basal optic root
ret. p.:	Nucleus reticularis pontis
rub. mag.:	Nucleus ruber, pars magnocellularis
rub. parv.:	Nucleus ruber, pars parvocellularis
s:	Lateral complex of the IVth and VIth tectal layers
SCO:	Subcommissural organ
t:	Ventral portion of the periaqueductal grey
u, v, w:	Portions of the pretectal region

Nuclei cited by GILLILAN but not demonstrated in the plates of this article (the Roman numerals indicate the plate at which the nucleus is mentioned in the text):
annular nucleus of the fasciculus longitudinalis medialis (II)
nucleus dorsalis lemnisci lateralis (II, III)
grey associated with the ansulate commissure (V)
nucleus centralis lateralis (III)
nucleus of the decussation of Forel (V)
nucleus trochlearis (IV).

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THE EFFECT OF CATIONS ON AMMONIA ACCUMULATION IN BRAIN CORTEX SLICES

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VARIOUS physiological states of an individual are accompanied by changes in the ammonia content of the central nervous system (VLADIMIROVA, 1938; RICHTER and DAWSON, 1948) as well as by alterations in the concentration of ions (KASSIL and PLOTITSINA, 1936). It is therefore of interest to test whether an interrelationship exists between these two phenomena. The effect of potassium ions on ammonia accumulation in brain cortex slices of guinea pigs, using no substrate in the incubation medium has already been studied (VRBA, FOLBERGROVÁ and KANTŮREK, 1958).

In the work reported in this paper, the effect of potassium and calcium ions on the release of ammonia from brain slices was examined in the presence of glucose as substrate, i.e. under physiologically more favourable conditions for surviving tissue. In addition, the effect of rubidium and lithium ions on this process was investigated in the hope that the results of these experiments would lead to a clearer understanding of the mechanism of the action of potassium. It is known that potassium and calcium ions bring about changes in metabolic processes requiring a supply of energy. As examples, the influence of these ions on the Pasteur effect (ASHFORD and DIXON, 1935; DICKENS and GREVILLE, 1935), on the phosphocreatine level (MCILWAIN, 1952), on the incorporation of inorganic phosphate into organic phosphate fractions (FINDLAY, MAGEE and ROSSITER, 1954) and on glycogen formation (KLEINZELLER and RYBOVÁ, 1957; RYBOVÁ, to be published) may be cited. In view of this, the effect of 2:4-dinitrophenol, which is known to inhibit the formation of high-energy phosphate bonds, and the effect of anaerobiosis on ammonia accumulation in cortex slices *in vitro* were also studied.

METHODS

Guinea pigs of the same breed and of 300 g mean wt. were used. The brain was removed from the animal, treated as described by MCILWAIN (1951) and from each hemisphere four slices of the gray matter were prepared by the method of DEUTSCH (1936).

The slices were incubated for 4 hr at 37° in a Warburg apparatus either in 25 ml Erlenmeyer flasks or, where the Q_{O_2} was measured, in Warburg vessels of the usual type. An incubation time of 4 hr was chosen as the amount of NH_3 in the medium at that time differed markedly from the initial value. In individual experiments brains of 3 animals were always used. Six incubation flasks were set up, each containing 3 ml of the appropriate medium and three slices from various cortical layers. The mean wt. of each slice was 60–70 mg; the total wt. of tissue in each flask was about 200 mg. The initial content of ammonia was determined in one slice from each hemisphere.

Krebs–Ringer phosphate of pH 7·4 was used as the incubation medium, with oxygen as the gaseous phase. Glucose (0·3% w/v) was added in experiments performed in the presence of substrate. When the effects of potassium, rubidium and lithium ions were investigated, the medium was modified by replacing the appropriate amount of NaCl by an equivalent amount of KCl, RbCl or LiCl. Where the effect of calcium ions was studied the $CaCl_2$ was replaced by an equivalent amount of NaCl;

the influence of an increased concentration of Ca^{++} was not examined because the corresponding medium could not be prepared without substantially decreasing the phosphate concentration and thus causing a marked diminution of the buffering capacity.

The ammonia which accumulated was estimated according to CONWAY (1939). The incubation of slices was stopped by adding 0·5 ml 10% (w/v) trichloroacetic acid; the contents of the flasks were quantitatively transferred to centrifuge tubes and the volume made up to 5 ml with distilled water. All these procedures were performed at 0° in order to prevent the breakdown of glutamine (RICHTER and DAWSON, 1948). After centrifugation, 2 ml of the supernatant were pipetted into the outer compartment of Conway vessels for ammonia estimation. Ammonia released on adding saturated K_2CO_3 and absorbed overnight at room temperature in boric acid in the inner chamber of the vessel, was estimated by titration with 0·02 N-HCl. The S.E. of this estimation was ±1·13%. No correction was made for the amount of NH_3 released from glutamine present in the medium, this amount being small and thus not appreciably affecting the results.

The results, shown in the Figure and in the Tables, represent means of at least four experiments, in which estimations in different media were carried out in duplicate or triplicate. All values are expressed as μmoles NH_3/g fresh wt.

RESULTS

Effect of potassium, rubidium and lithium ions on ammonia accumulation. If the ammonia formation in brain cortex slices is investigated in relation to the potassium ion concentration of the medium, with added substrate, the curve shown in Fig. 1 is

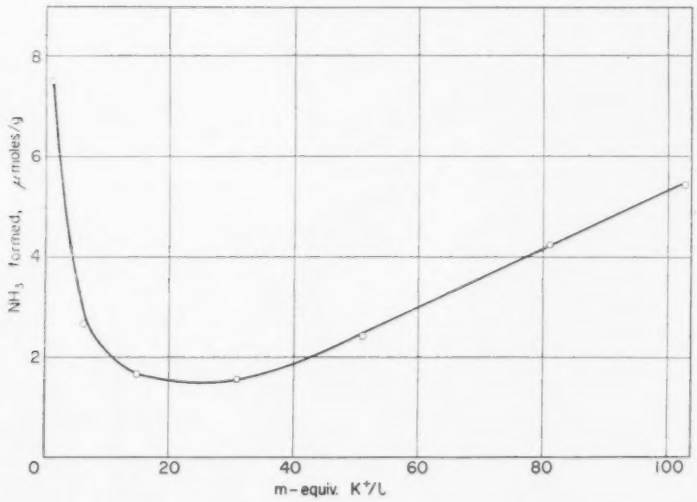

FIG. 1.—The effect of varying concentrations of K^+ on ammonia formation in brain cortex slices. Slices incubated aerobically 4 hr at 37° in Krebs–Ringer phosphate containing 0·3% (w/v) glucose. Ammonia was determined in portions of the total vessel contents and is expressed in μmoles/g fresh weight; all results being statistically significant at $P < 0·01$.

obtained. At a concentration of 1·25 m-equiv.K^+/l. medium (KCl in the Krebs-Ringer phosphate replaced by equivalent amount of NaCl) 7·5 μmoles NH_3/g wet wt. accumulated during incubation. In a medium with the usual ionic composition the amount of ammonia accumulated was considerably lower (2·66 μmoles/g) and on increasing the potassium ion concentration up to 25 m-equiv./l. a further decrease in ammonia formation was observed. If higher concentrations of potassium were used, ammonia production rose gradually. At a concentration of about 55 m-equiv. K^+/l.

the same amount of ammonia was formed as with the Krebs–Ringer phosphate. On replacing all the NaCl by KCl the quantity of ammonia accumulated increased up to 9·5 μmoles/g. The values plotted in the graph represent the quantity of ammonia formed during incubation; they were obtained by subtracting the initial amount of ammonia (on the average 3·2 μmoles/g) from the total amount of ammonia present in the medium after incubation.

Because the question arose as to whether this effect is specific for potassium ions or whether cations with similar properties have the same effect, the actions of rubidium and lithium ions were studied.

It is already known that many metabolic processes affected by potassium ions are similarly influenced by rubidium and lithium. The presence of these ions in the medium brings about an increase in Q_{O_2} (DICKENS and GREVILLE, 1935;), an impairment of glycogen synthesis (RYBOVÁ, to be published) and an increase in the swelling of brain cortex slices (PAPPIUS, ROSENFELD, MCLEAN JOHNSON and ELLIOTT, 1958; RYBOVÁ, to be published).

TABLE 1.—THE EFFECT OF RUBIDIUM AND LITHIUM IONS ON AMMONIA ACCUMULATION IN BRAIN CORTEX SLICES

Modification of medium	NH_3 content				Q_{O_2}		
	Initial	Controls	50 m-equiv. Rb^+ or Li^+/l.	100 m-equiv. Rb^+ or Li^+/l.	Controls	50 m-equiv. Rb^+ or Li^+/l.	100 m-equiv. Rb^+ or Li^+/l.
A. with added substrate (0·3% *glucose* w/v)							
Rb	3·1 (2·6–3·4)	5·4 (4·9–6·2)	4·3 (3·9–4·6)	8·8 (8·3–9·8)	—9·15	—17·8	—13·5
Li	3·4 (2·8–3·8)	6·8 (5·4–7·6)	7·7 (6·7–8·6)	9·1 (8·3–10·3)	—8·7	—11·2	—13·9
B. without added substrate							
Rb	3·6 (2·8–4·1)	29·2 (27·9–30·8)	19·5 (18·6–20·2)	16·4 (15·0–17·6)	—6·15	—3·59	—3·37
Li	3·9 (3·4–4·4)	28·5 (27·8–29·2)	24·0 (22·4–26·9)	23·1 (22·4–24·1)	—5·75	—4·89	—4·83

Slices incubated aerobically 4 hr at 37° in Krebs–Ringer phosphate. Ammonia was determined in portions of the total vessel contents and is expressed in μmoles/g fresh wt. All results are statistically significant at $P < 0{\cdot}01$, except the results of 50 m-equiv. Li^+/l. with added substrate (significant at $P < 0{\cdot}02$).

It follows from Table 1 that the influence of rubidium ions on ammonia accumulation was analogous to the effect of potassium ions; raising the concentration of rubidium ions to 50 m-equiv./l. produced an even more marked decrease in the amount of ammonia formed. When the amount of lithium in the incubation medium was increased (50 m-equiv. Li^+/l.) no decrease in ammonia accumulation could be observed. Even in media with lower concentrations of lithium, not included in the Table, no lowering of the amount of released ammonia as compared with the controls was observed, an increase being apparent at concentrations above 40 m-equiv. Li^+/l.

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VRBA, FOLBERGROVÁ and KANTŮREK (1958) have studied the effect of potassium ions on ammonia formation in the absence of substrate and have found that potassium depressed the quantity of ammonia produced.

As shown in Table 1, the effect of rubidium and to a lesser extent that of lithium is analogous to the influence of potassium on the amount of ammonia formed in the course of incubation of brain cortex slices in a medium without substrate. Q_{O_2} values presented in Table 1 were calculated on the basis of measurements taken for the first 90 min of incubation.

Effect of calcium on ammonia accumulation. The antagonism of the effects of calcium and potassium ions on some metabolic processes of the brain cortex, e.g.

TABLE 2.—THE EFFECT OF Ca^{++} ON AMMONIA ACCUMULATION IN BRAIN CORTEX SLICES

Substrate	NH_3 content		
	Initial	Controls	No Ca
None	3·1 (2·7–3·4)	27·2 (26·0–27·9)	24·3 (23·1–24·8)
Glucose	2·7 (2·3–3·1)	4·4 (3·8–5·2)	8·1 (7·6–9·2)

Slices incubated aerobically 4 hr at 37° in Krebs–Ringer phosphate. Ammonia was determined in portions of the total vessel contents and is expressed in μ moles/g fresh weight; all results being statistically significant at $P < 0{\cdot}01$.

TABLE 3.—THE EFFECT OF 2:4-DINITROPHENOL AND ANAEROBIOSIS ON AMMONIA ACCUMULATION IN BRAIN CORTEX SLICES

NH_3 content				
Initial	Controls	5×10^{-4} M dinitrophenol	N_2 with glucose	N_2 without glucose
3·8 (3·5–4·4)	6·1 (5·5–7·6)	9·2 (8·8–11·7)	—	—
2·9 (2·6–3·5)	4·9 (4·0–5·6)	—	7·2 (6·0–7·8)	9·8 (9·1–10·4)

Slices incubated 4 hr at 37° in Krebs–Ringer phosphate. Ammonia was determined in portions of the total vessel contents and is expressed in μmoles/g fresh weight; all results being statistically significant at $P < 0{\cdot}01$.

respiration (DICKENS and GREVILLE, 1935), aerobic and anaerobic glycolysis (ASHFORD and DIXON, 1935) and the content of high-energy compounds (MCILWAIN, 1952) has already been reported. This antagonism was also observed in our experiments on ammonia accumulation (Table 2). In a calcium-free medium ammonia formation in the presence of substrate increased more than twofold as compared with the control while in the absence of substrate it was decreased. VRBA, FOLBERGROVÁ and KANTŮREK (1958) have already mentioned a decrease in the amount of ammonia formed

in media with neither Ca^{++} nor substrate; the authors maintain, however, that this decrease was not statistically significant.

Effect of anaerobiosis and 2:4-*dinitrophenol on ammonia accumulation.* Table 3 shows that both 2:4-dinitrophenol at a concentration of 5×10^{-4} M and anaerobiosis significantly increased the amount of ammonia formed. Under anaerobic conditions less ammonia was formed when glucose was present than when no substrate was added. The effect of 2:4-dinitrophenol on ammonia accumulation in the absence of added substrate is exactly the opposite, i.e. the accumulation of ammonia is significantly decreased (WEIL-MALHERBE and GREEN, 1955).

DISCUSSION

The experiments reported here show that in the presence of exogenous substrate the influence of potassium ions is almost opposite to that in the absence of added

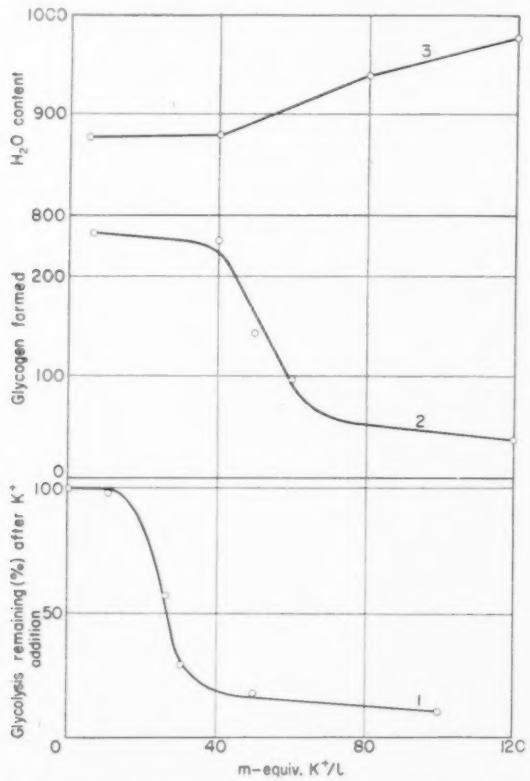

FIG. 2.—Comparison of the effect of varying concentrations of K^+ on (1) glycolysis (results expressed in % of anaerobic glycolysis in controls), (2) glycogen synthesis (results expressed in μg glucose/g fresh wt.) and (3) tissue hydration (results expressed in ml H_2O/100 g dry solids).

substrate (VRBA, FOLBERGROVÁ and KANTŮREK, 1958); on increasing the K^+concentration to 30 m-equiv./l. the amount of accumulated NH_3 in brain cortex slices decreased, but when the K^+ concentration was raised further the amount of NH_3 formed rose roughly linearly.

The effect of potassium ions on some metabolic processes in the central nervous system has already been studied in some detail. If the changes in the phenomenon studied are plotted against the concentration of potassium ions in the medium, a curve is obtained with a turning point at 20–40 m-equiv. K^+/l. Data on the effect of potassium on anaerobic glycolysis (DIXON, 1949), on glycogen synthesis and the swelling of brain slices during leaching (KLEINZELLER and RYBOVÁ, 1957) are given in Fig. 2.

High concentrations of K^+ *in vitro* impair energy-requiring reactions and lower the level of high-energy compounds in brain cortex. The described effect of K^+ on ammonia accumulation could also be explained on this basis. In the presence of glucose, the utilization of some endogenous nitrogenous substrate and consequently also the release of ammonia might be considerably decreased (VRBA and FOLBERGROVÁ, 1958), most of the NH_3 produced in the surviving tissue in the course of incubation would then be essentially of proteolytic origin. Under conditions unfavourable for protein regeneration (e.g. an insufficient supply of energy due to the high concentration of K^+ in the medium) it could be expected that more ammonia would be produced as a result of increased proteolysis and deamidation of proteins. This is in full agreement with our results. Furthermore, it must be borne in mind that in the presence of glucose, ammonia combines with the glutamic acid formed from glucose to give glutamine (KREBS, 1935). This energy-requiring reaction will also be suppressed in the presence of a high concentration of K^+, giving an increased amount of free ammonia in the slices. On these assumptions, 2:4-dinitrophenol would bring about similar results, as was obtained in our experiments.

Changes in the concentration of potassium in the medium alter the membrane potential (LI and MCILWAIN, 1957): the activated break-down of high-energy phosphate bonds located at (or near) the membrane might be considered as one of the mechanisms of potassium ion action (KLEINZELLER and RYBOVÁ, 1957). Furthermore a connection between a change in the ratio of bound to free intracellular potassium and different potential values can be assumed; it may consequently also be linked to changes of activity of some enzyme. Since this effect could exist only in a certain potential range the variability of the potassium effect would not be surprising (cf. Fig. 2).

It follows from experiments in which Rb^+ and Li^+ were used that the effect of K^+ is not specific. The similarity between K^+ and Li^+ is less marked, which is not unexpected as the physical properties of the lithium ions (e.g. diameter of the hydrated ion) differ considerably from those of the potassium ions.

The effect of calcium ions on metabolic processes in the nervous tissue was studied in more detail. It is generally assumed that most of the cell calcium is bound, probably in the membrane, and that the mechanism of its action is based on the changes it produces in the membrane potential (BRINK, 1954; FRANKENHAEUSER and HODGKIN, 1957). The omission of calcium ions from the medium causes a decrease in glycogen synthesis and an increase in brain tissue swelling in the course of leaching at 0°(RYBOVÁ, to be published), along with the increase in Q_{O_2} and anaerobic glycolysis (ASHFORD and DIXON, 1935; DICKENS and GREVILLE, 1935) and the decrease in phosphocreatine content of brain slices (MCILWAIN, 1952) already mentioned. Thus the effect of Ca^{++} appears to be antagonistic to that of K^+ in all the metabolic processes of grey matter investigated so far.

Since both the increase in potassium concentration and the decrease in calcium

concentration in the incubation medium bring about a swelling of brain cortex slices, dilution of enzymes and/or essential metabolites may be considered as a further cause of the metabolic effect of these ions.

SUMMARY

(1) The effect of an increased concentration of potassium ions in the presence of added substrate on ammonia accumulation in brain cortex slices was investigated. It was found that by increasing the K^+ concentration from 1·25 to 30 m-equiv./l. the amount of ammonia which accumulated decreased from 7·5 to 1·5 μmoles/g. On increasing the K^+ concentration above 30 m-equiv./l. ammonia formation was also increased.

(2) A similar effect was observed with rubidium and lithium ions.

(3) Rubidium and lithium ions at raised concentrations brought about a decrease in ammonia formation when no substrate was added.

(4) The omission of calcium ions from the medium caused a statistically significant decrease in the amount of ammonia which accumulated in the absence of substrate; an increase was obtained in the presence of substrate.

(5) In the presence of added substrate the quantity of ammonia which accumulated under anaerobic conditions or in the presence of 2:4-dinitrophenol was greater than in the controls.

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AMINO ACID METABOLISM IN EPILEPTOGENIC AND NON-EPILEPTOGENIC LESIONS OF THE NEOCORTEX (CAT)

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APPLICATION of γ-aminobutyric acid (GABA) to the surface of the mammalian cerebral cortex rapidly and reversibly abolishes negative components of potentials evoked by local cortical excitation and stimulation of various afferent pathways (PURPURA, GIRADO and GRUNDFEST, 1957*a*; PURPURA *et al.*, 1959). Intravenously injected GABA, even in relatively high concentrations (100 mg/kg), produces neither electrophysiological alterations similar to those observed following topical application (PURPURA, GIRADO and GRUNDFEST, 1957*b*), nor significant increments in GABA concentration of brain (ROBERTS and BAXTER, 1957; ELLIOTT and VAN GELDEN, 1958). It therefore seems that, as with one of its precursors, glutamic acid (SCHWERIN, BESSMAN and WAELSCH, 1950), under conditions approaching the normal state GABA cannot penetrate the intact blood brain barrier in amounts sufficient to affect the brain physiologically.

In contrast, when the cerebral cortex is sprayed with ethyl chloride, not only is the blood brain barrier locally destroyed (as evidenced by penetration of Evans blue dye) but under certain conditions an epileptogenic focus is also produced (MORRELL and FLORENZ, 1958). In such preparations, intravenous injection of GABA produces rapid suppression of spontaneous potentials in the epileptogenic focus (PURPURA, GIRADO, SMITH and GOMEZ, 1958*a*,*b*) and effects entirely similar to those observed following topical application.

The aforementioned results suggest the possibility of analysing the metabolic alterations in areas of cortex made epileptogenic by local freezing of its surface. In the present study attempts have been made to correlate biochemical observations with electrophysiological and pharmacological phenomena. The results of the initial investigation indicate that decreases in concentration of glutamic acid, glutamine and glutathione were dependent on the alterations produced in the lesion areas but that no significant changes in GABA concentrations were observed.

MATERIALS AND METHODS

Unanaesthetized succinylcholine-paralysed adult cats (2–4 kg) prepared as described previously by PURPURA and GRUNDFEST (1956, 1957) were employed in all experiments. At least 45–90 min were allowed for recovery from all operative procedures before the posterior sigmoid gyrus (left somatic-sensory cortex) was treated with a spray of ethyl chloride (cf. MORRELL and FLORENZ, 1958). Prior to and immediately after the production of such freezing lesions, electrocortical activity was constantly monitored from the lesion site and homologous regions in the opposite hemisphere.

Uninjured tissue from the contralateral cortex was used as the control against which changes in

amino acid concentrations in injured areas were compared. Sites immediately adjacent to the lesion area were also analysed in two preparations.

At varying times after the production of lesions the experiments were terminated by rapid freezing of the exposed brain with solid CO_2. At the time of application of dry ice the animals were also injected with a lethal dose of pentabarbital sodium. The cortex (75–125 mg) was removed in the frozen state, trimmed of white matter, and maintained frozen until extracted with cold 5% (w/v) trichloroacetic acid.

Glutamic acid, glutamine, GABA and glutathione were isolated and determined by the methods of BERL and WAELSCH (1958).*

RESULTS

Paroxysmal activity similar to that shown in Fig. 1 developed in approximately two thirds of the animals within 30–60 min after production of freezing lesions. In approximately one third of the animals similar lesions did not become epileptogenic

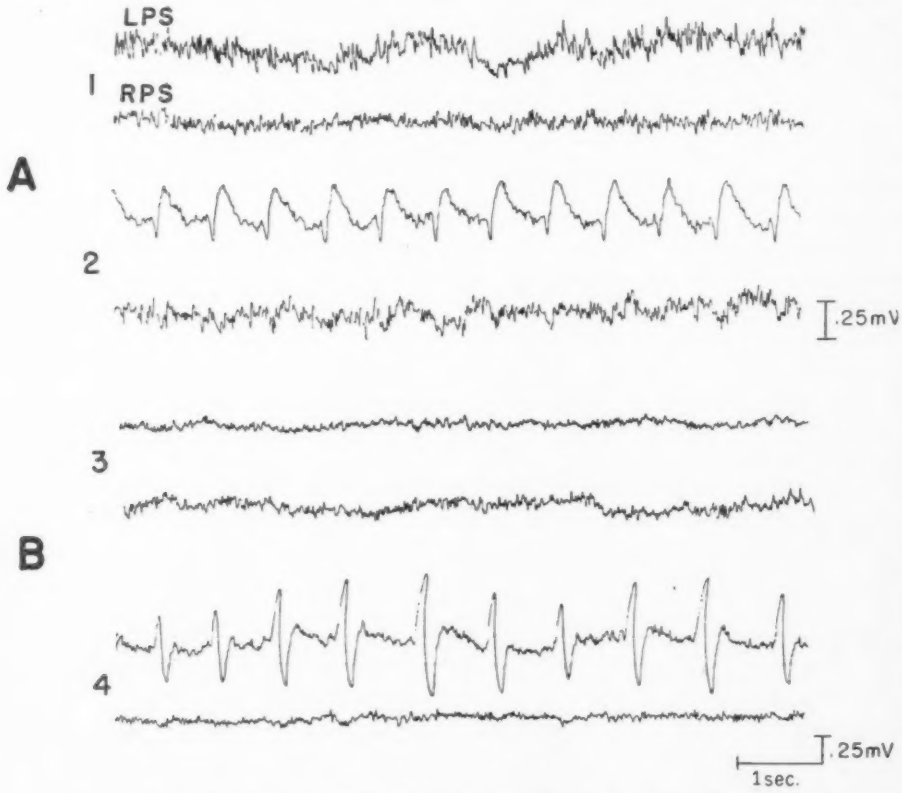

FIG. 1.—Electrographic characteristics of focal spike discharges developing in epileptogenic lesions of somatic sensory cortex. In each set upper record is from left posterior sigmoid gyrus (LPS), and lower tracing right posterior sigmoid gyrus (RPS). Activity recorded bipolarly in A and monopolarly in B (indifferent electrode consisting of a screw imbedded in bone overlying the frontal sinus). Negativity upwards in B.A. 1. Controls prior to production of freezing lesion in LPS. 2. 45 min later, immediately after development of focal discharges. B. 3. Controls in another preparation prior to freezing lesion in LPS. 4. Three hours after production of lesion, two hours after onset of focal discharges. Note absence of projected activity to contralateral cortex.

* 0·2 M-Ammonium citrate buffer, pH 3·1, was used instead of ammonium formate buffer for the elution of the glutamine from the Dowex 50 columns.

during the period of observation (3–4 hr), although identical techniques were used as far as can be judged. The latter group (4 animals), therefore, constituted one series of experiments which served as controls to differentiate the effects produced by lesions alone in the absence of paroxysmal activity. Experiments were terminated

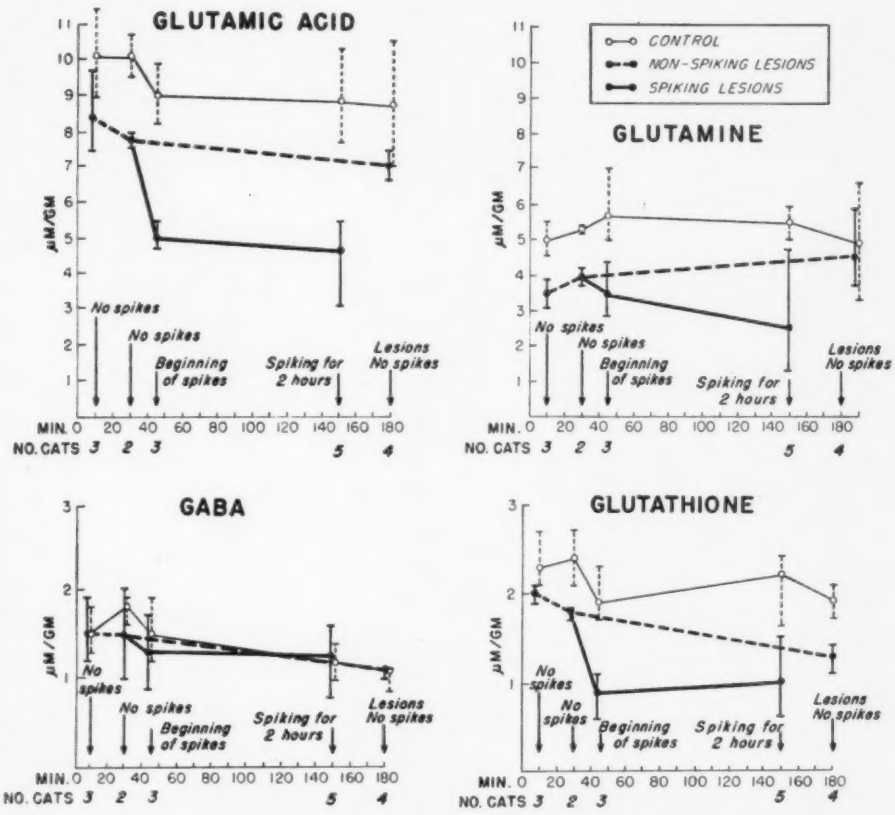

FIG. 2.—Results of an analysis of 34 samples of cortex in 17 cats. 'Control'r efers to samples taken from areas contralateral to lesion sites. All values expressed in µM/gm (ordinate); time after production of lesion (abscissa). Vertical bars represent the magnitude of the variation in each series of determinations. Lines are drawn through mean values. Note that changes in glutamic acid, glutamine and glutathione are not associated with changes in GABA concentration.

at the onset of spiking (3 animals) and after 2 hr of sustained low-frequency paroxysmal activity (5 animals). The immediate effects of the lesions were determined by sacrificing animals at 10 min (3 animals) and 30 min (2 animals) after application of the ethyl chloride spray, i.e. before the onset of paroxysmal activity. The results from the study of these 17 cats are presented in Fig. 2.

Lesion areas which developed focal paroxysmal discharges showed a decrease of 30–75 per cent in content of glutamic acid, glutamine and glutathione as compared with the contralateral control areas. This decrease became evident at the time of onset of the spikes, i.e. about 45 min after the production of the lesion. The fall in

level of glutamic acid, glutamine and glutathione was much less pronounced in lesions which did not develop detectable paroxysmal activity.

Of particular interest was the finding that irrespective of the characteristics of the lesions, duration of the experiment, or intensity of paroxysmal activity, no significant reduction in GABA concentration was detectable as compared with control areas.

In two animals cortex tissue adjacent to the lesion was also removed and analysed (Table 1); the lesions showed decreases in glutamic acid, glutamine and glutathione

TABLE 1.—ETHYL CHLORIDE LESION IN POSTERIOR SIGMOID GYRUS OF THE CAT

Cortical area	μmoles/g Tissue			
	Glutamic acid	Glutamine	GABA	Glutathione
Lesion	5·0	4·8	1·6	1·0
Contralateral	8·3	5·9	1·4	1·6
Adjacent to lesion	8·2	5·4	1·6	1·9
Lesion	5·5	1·4	1·4	0·9
Contralateral	10·3	5·1	1·2	2·4
Adjacent to lesion	10·7	4·9	1·1	2·5

Focal paroxysmal activity confined to the lesion site for approximately 2 hr. Cortex adjacent to the lesion was not involved in spread of the paroxysmal activity.

similar to those graphically presented in Fig. 2. One of these two animals failed to show change in glutamine greater than 20 per cent (Table 2). Areas immediately adjacent to the lesions did not show significant variation from contralateral cortex in the concentration of the compounds analysed.

DISCUSSION

The extensive changes in glutamic acid, glutamine and glutathione concentrations appear to occur simultaneously with the development of focal spikes and not before. Therefore, it is probable that the decreases in the tissue concentration of these metabolites are in some way associated with the development of focal paroxysmal activity in these particular lesions. However, it may be argued that such changes might reflect alterations which could result from augmented metabolism of the epileptogenic lesions. This seems unlikely in view of additional observations that localized cortical paroxysmal activity induced by low frequency electrical stimulation for a period of two hours did not significantly alter the concentration of the metabolites analysed above (BERL, PURPURA and WAELSCH, unpublished).

It is surprising that the GABA levels in the lesions showed no basic alteration from the control values. It is known from the work of ROBERTS and BREGOFF (1953) that glutamic acid is the precursor of GABA via decarboxylation. It is also known that GABA is rapidly metabolized in the brain (ROBERTS, ROTHSTEIN and BAXTER, 1958; LAJTHA, BERL and WAELSCH, 1959). It cannot be excluded at the present time that the characteristic of the epileptogenic lesion is a block in the metabolism of GABA whereas glutamic acid, glutamine, and the glutamic acid moiety of glutathione

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are metabolized via the citric acid cycle. It appears more likely that the GABA level is maintained at the expense of glutamic acid and its other metabolic derivatives. The decision between these alternatives will be obtained by the use of ^{14}C-labelled glutamic acid and GABA.

It is, of course, of major interest that the concentration of GABA in the lesion did not show any changes although a small amount administered topically, or a larger amount intravenously, suppressed evoked potentials. It should be kept in mind that the lack of a gross change in GABA concentration does not preclude a

TABLE 2.—PERCENTAGE DECREASE FROM CONTROL VALUES

	Number of animals in each percentage range					Average decrease
	0–19	20–29	30–39	40–59	60–79	
Glutamic acid						
Nonspiking lesion	3	5	1			19
Spiking lesion		1	1	4	2	46
Glutamine						
Nonspiking lesion	3	3	3			23
Spiking lesion	1		2	2	3	49
Glutathione						
Nonspiking lesion	3	1	5			25
Spiking lesion			3	1	4	53
GABA						
Nonspiking lesion	8		1			3
Spiking lesion	6	2				5

change in concentration at axodendritic synapses where presumably only a small fraction of the GABA determined by analysis of the whole tissue sample may be located. In support of such a possibility is the preliminary finding that after the administration of GABA the focal seizure activity is suppressed before the concentration of GABA is significantly altered.

Changes observed in glutamic acid concentration in the lesion with and without focal seizure activity cannot be readily correlated with the seizure activity, partly because the chemical analysis encompassed only a limited number of compounds. It should be noted that major changes in the concentration of glutamic acid, glutamine and glutathione coincide with the onset of the seizure activity whereas in those lesions without seizure activity an intermediate level of concentration of these compounds is maintained during the experimental period. One animal in the series with spiking lesions failed to develop a significant decrease in glutamine (Table 2). This would support the possibility of a preferential mobilization of glutamic acid although the results of the other experiments (Table 2) neither support nor disprove such a sequence of events. The question arises, therefore, whether or not a metabolic product of glutamic acid liberated and not removed in these lesions may be the agent responsible for seizure activity. In this respect interest centres on ammonia

whose relation to seizure activity has been suspected for a long time (WAELSCH, 1955). If the decrease of glutamic acid and glutamine concentrations results from metabolic degradation, the concentration of free ammonia should increase considerably, particularly since the dicarboxylic acid represents the substrate for the two major enzymic activities for insuring a low tissue level of free ammonia. Whether an additional mechanism for removal of ammonia by converting GABA to γ-guanidinobutyric acid operates in nervous tissue is not known although the possibility of a reversible transfer of the guanidino group between the latter compound and arginine is suggested by recent experiments (PISANO and UDENFRIEND, 1958). Such a mechanism may be overwhelmed by high ammonia concentration. Since it has been shown that γ-guanidinobutyric acid is capable of augmenting evoked surface negative potentials in neocortex, presumably by inactivating hyperpolarizing axodendritic synapses (PURPURA, GIRADO and GRUNDFEST, 1958), experiments are under way to define more clearly the role of γ-guanidinobutyric acid, ammonia and aspartic acid concentrations in epileptogenic lesions.

It should be noted that the percentage decrease of glutathione in the spiking and nonspiking lesion is at least as large as that of glutamic acid. Because of the role of glutathione in cell metabolism, particularly in reference of SH containing enzymes, it can not be excluded that its decrease is implicated in the abnormal metabolism of the lesion.

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SUMMARY

The concentrations of γ-aminobutyric acid, glutamic acid, glutamine and glutathione have been determined in epileptogenic lesions produced by restricted freezing of small areas of cortex in unanaesthetized succinylcholine-paralysed cats. The results are compared with the concentrations of these metabolites in non-epileptogenic lesions and intact cortex.

(1) The onset of focal seizure activity in lesion sites is associated with significant decreases in glutamic acid, glutamine and glutathione.

(2) Decreases in the concentrations of these compounds are greater in epileptogenic lesions than in non-epileptogenic lesions.

(3) Areas immediately adjacent to lesion sites have a normal content of these metabolites.

(4) No significant difference in GABA concentration was detectable in epileptogenic lesions, non-epileptogenic lesions and intact cortex.

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THE URINARY EXCRETION OF ADRENALINE AND NORADRENALINE BY SCHIZOPHRENIC PATIENTS DURING RESERPINE TREATMENT

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IN animal experiments several investigators have demonstrated the depletory action of reserpine on the stores of catecholamines (CARLSSON and HILLARP, 1956; BERTLER, CARLSSON and ROSENGREN, 1956; HOLZBAUER and VOGT, 1956; BRODIE, OLIN, KUNTZMAN and SHORE, 1957; CARLSSON, ROSENGREN, BERTLER and NILSSON, 1957; STJÄRNE and SCHAPIRO, 1958). In patients treated with reserpine BURGER (1957) found a decrease in blood noradrenaline, and GADDUM *et al.* (1958) reported a diminished excretion in urine of adrenaline and noradrenaline.

As part of an investigation of the excretion of catecholamines in mental patients under treatment with various drugs, a group of male schizophrenic patients under reserpine treatment were examined. The dosage of reserpine varied from 1 to 9 mg daily, usually 3 mg. Duration of treatment: 6 to 45 months.

METHODS

Determination of catecholamines in urine. The urine was collected for 24 hr in bottles containing 200 ml N-perchloric acid. One hundred mg ethylene diamine tetraacetic acid (disodium salt) and 5 mg ascorbic acid were added to two urine samples of 10 ml each. To one sample was added 2 μg noradrenaline in order to check the recovery. (Recoveries of both adrenaline (A) and noradrenaline (NA) are usually about 80 per cent.) By means of a Beckman-titrator or bromthymol blue the samples were neutralized to pH 7, centrifuged, and passed through a Dowex 50, X-8, column (200–400 mesh, dimensions 5·5 × 15 mm), loaded with 0·1 M-phosphate buffer, pH 6·5. The flow rate was kept at 0·5–1 ml/min. The column was washed with 40 ml distilled water. A and NA were eluted with 7·0 ml N-HCl. The eluates were neutralized to pH about 6·5 (checked with indicator paper) by means of 5N-K_2CO_3, and A and NA were determined fluorimetrically after oxidation and subsequent rearrangement to adrenolutine and noradrenolutine, using an Aminco-Bowman spectrophotofluorometer. The different activation peaks of the fluorophores were utilized for differentiation between A and NA. Further details of the procedure are given by BERTLER *et al.* (1958).

RESULTS

The results obtained appear in Tables 1 and 2. Long-term oral administration of reserpine to 11 schizophrenic patients was followed by a marked decline in the rate of excretion of noradrenaline, while the excretion of adrenaline was unchanged compared to control values obtained from 13 untreated schizophrenic patients. No antidiuretic effect of long-term reserpine treatment could be demonstrated.

Three months after discontinuation of the reserpine treatment the values of noradrenaline in 3 cases rose respectively from 3 to 34, 6 to 38, and 3 to 29 μg/24 hr. In 2 cases the corresponding values rose from 3 to 5 μg/24 hr and 3 to 6 μg/24 hr, respectively, 3 days after discontinuation of the treatment.

TABLE 1.—RESERPINE-TREATED SCHIZOPHRENIC PATIENTS

Patient No.	Age	Volume of urine (ml)	Adrenaline (μg/24 hr.)	Noradrenaline (μg/24 hr.)
1	39	1430	19	8·9
2	45	1500	14	0
3	67	1070	5·6	6·5
4	54	1975	94	0
5	55	1300	14	3·9
6	52	1600	9·6	11
7	71	1600	20	0
8	44	1785	7·9	4·6
9	54	1400	89	0
10	61	800	6·4	3·1
11	63	2100	13	11
		Mean: 1505	Mean: ± S.E.M. 26·6 ± 9·8	Mean: ± S.E.M. 4·5 ± 1·3

No correlation could be demonstrated between the clinical effect of the reserpine treatment and the diminished excretion of noradrenaline. In the control material of untreated schizophrenic patients the ratio of adrenaline to noradrenaline excretion was 1 : 1·3.

DISCUSSION

In contrast to these findings GADDUM *et al.* (1958), using a fluorimetric method after chromatographic separation of the amines, demonstrated a decrease in the values of both adrenaline and noradrenaline during long-term reserpine treatment. In the experiments reported here a decrease in the noradrenaline values was found, while the adrenaline excretion appeared unchanged. The antidiuretic effect of reserpine demonstrated by GADDUM *et al.* (1958) was not confirmed in the present investigation. The somewhat higher average doses of reserpine used by the latter workers may possibly account for these differences in results.

SUMMARY

The urinary excretion of adrenaline and noradrenaline was estimated in 11 chronic schizophrenic patients during long-term oral reserpine treatment and in 13 untreated chronic schizophrenic patients. The values of noradrenaline were markedly lower in the former group, while no difference in the excretion of adrenaline was detectable. No antidiuretic effect of the reserpine treatment was observed.

TABLE 2.—UNTREATED SCHIZOPHRENIC PATIENTS

Patient No.	Age	Volume of urine (ml)	Adrenaline (μg/24 hr)	Noradrenaline (μg/24 hr)
12	56	800	18	14
13	19	800	19	34
14	54	1025	20	16
15	39	690	6·0	19
16	61	1080	63	66
17	46	2220	31	68
18	47	1860	15	0
19	47	1080	18	24
20	58	1170	33	32
21	36	700	10	27
22	48	1020	46	65
23	40	3370	23	37
24	40	1260	23	31
		Mean: 1314	Mean: ± S.E.M. 25·0 ± 4·2	Mean: ± S.E.M. 33·3 ± 5·9
			$P > 0.05$ difference not significant	$P < 0.01$ difference significant

Acknowledgements—Technical assistance was given by Mrs. BERIT ANDERSSON and Miss MARY KÜTT. The investigation was supported by a generous grant from The Swedish Ciba, Ltd., Stockholm.

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THE URINARY EXCRETION OF ADRENALINE AND NORADRENALINE BY DEPRESSIVE PATIENTS DURING IPRONIAZID TREATMENT

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*Iso*propyl-*iso*nicotinic acid hydrazide (iproniazid, Marsilid) is known as a potent inhibitor of monoamine oxidase (MO) *in vitro* (ZELLER, BARSKY, FOUTS, KIRCHEIMER and VAN ORDEN, 1952) and *in vivo* (ZELLER and BARSKY, 1952; SCHAYER and SMILEY, 1953). MO is able to inactivate sympathomimetic amines by oxidative deamination. SCHAYER and SMILEY (1953) and SCHAYER *et al.* (1955), using ^{14}C-labelled adrenaline and noradrenaline, concluded that MO was responsible for the catabolism of a major part of these substances. In this connexion it should be mentioned that the results of SCHAYER do not agree with investigations of VON EULER and HELLNER-BJORKMAN (1955), VON EULER and ZETTERSTRÖM (1955) and CORNE (1956), using other methods of investigation. RESNICK *et al.* (1958), examining the urine following infusion of ^{14}C-labelled adrenaline, stated that iproniazid in schizophrenic patients and at a dose level of 100–150 mg/day inhibited amine oxidase activity by approximately 50 per cent, but did not influence the O-methylation of adrenaline described by AXELROD (1958) and LA BROSSE, AXELROD and KETY (1958).

These authors conclude that O-methylation is the principal pathway of metabolism of adrenaline as well as of noradrenaline and suppose that catechol-O-methyl transferase is the principal enzyme for the inactivation of adrenaline and noradrenaline. The rate of O-methylation of noradrenaline in the brain was found to be the same in normal and iproniazid treated rats, but AXELROD (1958) demonstrated that the ability of brain mitochondria prepared from animals pretreated with iproniazid to metabolize normetanephrine (3-methoxynoradrenaline) was markedly reduced. The stimulating and antidepressive effect of iproniazid, observed clinically, is believed to be connected with accumulation of catecholamines and serotonin in brain tissue (PLETSCHER, 1957; SHORE, 1958).

METHODS

In order to study the influence of iproniazid treatment on the rate of excretion of catecholamines, 24 hr urine samples were collected from male depressive patients, mainly with endogenous and neurotic depressions. The dosage of iproniazid varied from 25 mg to 150 mg, usually 100 mg daily. Urine samples were analyzed before treatment, and after 8–33 days of iproniazid.

The *method* of determination of catecholamines in urine has been described in the preceding paper (*J. Neurochem.* **4**, 318).

RESULTS AND DISCUSSION

Table 1 shows the excretion of adrenaline and noradrenaline in 19 untreated patients, Table 2 in 12 patients given iproniazid. The means are not significantly

TABLE 1. —UNTREATED DEPRESSIVE PATIENTS
(total number: 19)

Patient no.	Age	Volume of urine (ml)	Adrenaline (μg/24 hr)	Noradrenaline (μg/24 hr)
1	44	1625	84	51
2	47	610	20	26
3	30	2600	36	30
4	41	740	23	86
5	51	1625	14	33
6	34	1000	16	18
7	39	1010	24	32
8	26	2500	28	27
9	67	2700	9·6	134
10	36	930	23	33
11	52	2100	22	60
12	45	640	13	17
13	39	2350	12	46
14	41	1650	31	54
15	65	1175	10	19
16	33	2200	22	49
17	46		23	84
18	47	3100	35	30
19	60	1050	13	27
		Mean: (1–19) 1644	Mean: ± S.E.M. 24·1 ± 3·8	Mean: ± S.E.M. 45·1 ± 6·8
		Mean: (1–8) 1464	30·6 ± 8·0	37·9 ± 7·6

TABLE 2.—IPRONIAZID TREATED DEPRESSIVE PATIENTS
(total number: 12)

Patient no.	Age	Volume of urine (ml)	Adrenaline (μg/24 hr)	Noradrenaline (μg/24 hr)
1	44	2130	23	57
2	47	625	6·9	19
3	30	3400	29	90
4	41	1250	13	87
5	51	1825	8·9	4·6
6	34	850	8·9	43
7	39	1600	21	22
8	26	3500	30	49
20	39	1425	9·7	20
21	30	3180	43	11
22	51	2150	20	24
23	57	1660	5·5	46
		Mean: (1–23) 1966	Mean: ± S.E.M. 18·2 ± 3·2	Mean: ± S.E.M. 39·4 ± 8·1
		Mean: (1–8) 1898	17·6 ± 3·3	46·5 ± 11·0

different. Neither is there a significant difference in the excretion of amines by the same patients (nos. 1–8) examined before and during administration of iproniazid.

From this investigation no conclusions could be drawn about the pathways of metabolism of adrenaline and noradrenaline in the human organism. In order to solve this problem it is necessary to isolate the intermediate and final products derived from the substances.

SUMMARY

In a group of depressive patients (endogenous depressions and neurotic depressions) no significant change in the rates of excretion of adrenaline and noradrenaline could be detected during iproniazid treatment.

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THE EFFECT OF INSULIN ON THE FORMATION OF PHOSPHORYLCHOLINE AND PHOSPHORYLETHANOLAMINE IN THE BRAIN

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IT was shown by DAWSON and RICHTER in 1950 that the uptake of [^{32}P]-orthophosphate into brain phospholipids *in vivo* was depressed in insulin hypoglycaemia. ANSELL and DOHMEN (1957) measured the depression in individual phospholipids, and showed that the depression covered phosphatidyl choline, phosphatidyl ethanolamine (and presumably the ethanolamine-containing plasmalogen), and diphosphoinositide. In an attempt to locate the point of action in the phospholipid synthetic chain the effect of insulin on the uptake of ^{32}P into phosphorylethanolamine and phosphorylcholine has been measured.

METHODS

Albino rats of 40 to 60 g body weight (4–6 weeks old) were fasted for 24 hr and injected intraperitoneally with 4 mc/kg body weight [^{32}P]-orthophosphate (carrier orthophosphate was also present). Plain insulin (40–120 units/kg body weight) was injected intraperitoneally into the animals 30 min before the isotope; the animals were either comatose or very depressed after 2 hr. After a three-hour isotopic exchange period the animals were killed, the brain tissue rapidly removed, homogenized in 10 ml of 12% ice-cold trichloroacetic acid (TCA), centrifuged and total lipids extracted from the washed precipitate as previously described (ANSELL and DOHMEN, 1956).

To 8 ml of the original TCA extract 400 mg of acid-washed charcoal ('Norit' A) was added to remove nucleotides (cf. CRANE and LIPMANN, 1953; THRELFALL, 1957) and the nucleotide-free extract plus washings then extracted with successive volumes of ether until the pH was about 4. Saturated barium hydroxide solution was added until the pH was 8·5 and the precipitate of the barium phosphate allowed to form for 15 min at 0°. This inorganic phosphate was partly the free inorganic phosphate in the tissue and partly derived from creatine phosphate and adenosine polyphosphates. Barium phosphate was removed by centrifuging. The water-soluble barium salts of the remaining phosphate esters were converted into their ammonium salts by shaking with Amberlite IR-120 (NH^+_4) (cf. HEALD, 1956) and the solution taken to dryness under reduced pressure at a temperature $<50°$. It was then subjected to two-dimensional paper chromatography on Whatman No. 1. paper (previously freed from cations with 2 N-acetic acid and distilled water and dried) using ethanol-18 N-ammonium hydroxide-water (60:30:10 v/v) (DAWSON, 1956) and phenol-18 N-ammonium hydroxide-water (80 g phenol + 0·3 ml of 18 N-ammonium hydroxide solution + 20 ml water) in that order. The use of the ethanol-ammonia solvent as the first solution ensured that some interfering ammonium salts in the extract (e.g. ammonium trichloroacetate) moved to the bottom of the paper whilst the phosphate esters migrated more slowly. On spraying the chromatogram with the reagent of HANES and ISHERWOOD (1949), drying at room temperature and developing under ultraviolet light, three prominent phosphorus compounds appeared in addition to a spot corresponding to inorganic phosphate which was not completely removed by the barium precipitation (Fig. 1). Two other fainter spots appeared which were presumably glycerylphosphorylethanolamine and glycerylphosphorylcholine; these moved faster than the monoesters in ethanol-NH_3. Heating the papers after spraying with the molybdic acid reagent was not found to be necessary.

Determinations of ^{32}P and ^{31}P on lipid samples and compounds separated by chromatography were carried out as previously described (ANSELL and DOHMEN, 1957).

Phosphorylcholine was prepared as the calcium salt of phosphorylcholine chloride by the method of PLIMMER and BURCH (1937); this was conveniently converted to the free ester by passing it over a column of Amberlite IRC-50(H^+) resin. Phosphorylethanolamine and phosphorylserine were obtained from the California Foundation for Biochemical Research, Ltd.

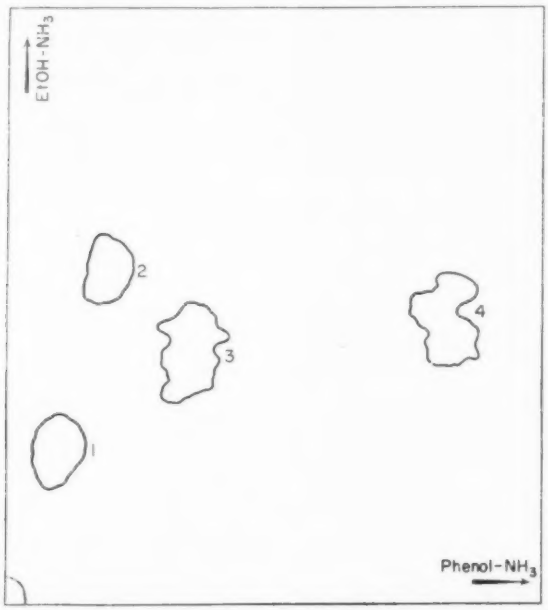

FIG. 1.—A radioautograph of a chromatogram of brain phosphate esters after the separation described in the text. (1) Inorganic phosphate; (2) glycerophosphate; (3) phosphorylethanolamine; (4) phosphorylcholine. Glycerylphosphorylethanolamine and glycerylphosphorylcholine, which travel much further in ethanol-NH_3, were not sufficiently radioactive to be visible.

RESULTS AND DISCUSSION

Phosphorylcholine, phosphorylethanolamine and glycerophosphoric acid in brain. Phosphorylcholine has been identified and estimated in brain tissue by DAWSON (1955*b*) and further determinations made in rat and hen nervous tissues by PORCELLATI (1958). Phosphorylethanolamine has been determined by ANSELL and DAWSON (1951) and PORCELLATI (1958) among others. For the purpose of determining the specific radioactivity, however, it was essential to know that these two phosphate esters separated by the present procedure were uncontaminated with other phosphorus-containing compounds.

Accordingly the two compounds located with an autoradiograph of an unsprayed chromatogram were eluted with water and the amount of ^{32}P present determined. To the phosphorylcholine eluate, reduced in volume to about 3 ml, was added 75 mg of the calcium salt of phosphorylcholine chloride. It was then crystallized repeatedly from water by the careful addition of ethanol and the specific radioactivity (counts/min/mg calcium salt) determined (DAWSON, 1955*b*) when it was shown by the constant specific radioactivity after several crystallizations that all the radioactivity in the

eluate was due to the phosphorylcholine. With phosphorylethanolamine a similar procedure was adopted using 110 mg of the free ester which for final confirmation was converted to the flavianate (ANSELL and DAWSON, 1951). It was clear therefore that the separation of the esters was adequate for the determination of specific radioactivity.

The average amount of phosphorylcholine found in the whole brain of 24 rats was 31 ± 10 μg/g brain but this value was considered low because experiments in which 120 μg phosphorylcholine was added to the TCA extracts prior to fractionation showed that the recovery was 49 ± 11 per cent (8 animals). In further experiments extracts from animals injected with ^{32}P were divided into two halves, to one of which was added 120 μg phosphorylcholine and the specific radioactivities of the undiluted and diluted phosphorylcholine were measured after fractionation. In each experiment the amount present in the tissue was calculated from the expression

$$\frac{b}{(sr_1/sr_2) - 1}$$

(where b = amount, sr_1 = the specific radioactivity of the undiluted sample, and sr_2 = the specific radioactivity of the diluted sample) and also from the actual recovery of added ester. The amount calculated on the basis of recovery from rat brains was $50{\cdot}8 \pm 8{\cdot}2$ μg/g brain and on the basis of 'isotope dilution' $56{\cdot}6 \pm 7{\cdot}4$ μg/g brain. As there was no indication that freezing the brain of the animal *in situ* in liquid oxygen gave different values the true level of phosphorylcholine in the whole brain tissue of 4–6 week old rats is probably 50–60 μg/g brain. This level is somewhat lower than found by DAWSON (1955*b*) (81 μg/g) and PORCELLATI (1958) (45–83 μg/g) although these workers were using much older animals. For 3–13 day-old animals MIANI (1957) found values of 450 μg/g which would indicate a precipitous decline in the cerebral content of the ester during the myelination period. This author has also reported high values for the phosphorylethanolamine, glycerylphosphorylethanolamine and glycerylphosphorylcholine in the brains of very young rats.

Estimation of the phosphorylethanolamine present in rat brain gave a mean value for 24 rats of 134 ± 32 μg/g brain which agrees with previous values reported by ANSELL and DAWSON (1951) and PORCELLATI (1958). It should be pointed out however that experiments indicated that recovery was only of the order of 70 per cent so that the true value may be somewhat higher.

The other prominent spot on the chromatogram (other than inorganic phosphate which amounted to 10–13 μg P/g brain, indicating that 95 per cent had been removed), was provisionally identified as glycerophosphate from the position and its inseparability from added $\alpha + \beta$-glycerophosphate. The relative position of glycerophosphate on chromatograms found in these experiments is similar to that obtained by FREINKEL (1958) in studies on the water-soluble phosphate esters of thyroid tissue. Elution of the spot from an unsprayed chromatogram and subsequent periodate oxidation by the method of ANSELL and NORMAN (1956) for determining α-glycerophosphate indicated that about 70 per cent of the phosphorus was liberated as inorganic phosphorus. If the compound is glycerophosphate it is likely that this would occur in the brain exclusively as the α-isomer. It is probable that partial conversion to the β-form had taken place during the separation from the original extract. DAWSON (1955*c*) and PORCELLATI (1958) identified free glycerophosphate in liver and brain extracts but

did not give values for the amount present. Estimation of the P in the glycerophosphate spot gave values for the ester of 31±5 μg glycerophosphate/g brain though this is a minimal value as there were considerable losses when glycerophosphate was added to the original TCA extract of the tissue and carried through the procedure. It is also possible that breakdown of glycerylphosphorylethanolamine and glycerylphosphorylcholine to glycerophosphate took place during the fractionation process. A contribution from this source would however be expected to lower the specific radioactivity considerably as these esters are enzymic breakdown products of phospholipids (DAWSON, 1955*a*) and of low specific radioactivity after a short exchange period. In fact, the specific radioactivity of the glycerophosphate 3 hr after injection was slightly higher than that of the acid-soluble fraction as a whole indicating that there is a rapid formation of this ester *in vivo*. Earlier estimations of free glycerophosphate in brain extracts by LEVA and RAPAPORT (1943) gave higher values, but these estimations are now known to be erroneous because the alcohol-insoluble fraction of the barium salts of phosphate esters which was considered to be glycerophosphate contains other phosphorus compounds (OLLEY and BLEWETT, 1950).

Two workers, PORCELLATI, (1958) and MIANI (1957) have located phosphorylserine on paper chromatograms of extracts of brain tissue. No spot corresponding to this compound was consistently observed in the present experiments although the amount found in rat brain by PORCELLATI (1958) was equivalent to 6–7 μg P/g tissue, and should have been visible.* MIANI (1957) found an amount of phosphorylserine equivalent to 29 μg P/g brain in very young rats (3–13 days old) and for rat liver an amount equivalent to 1·2 μg P/g tissue has been reported (NEMER and ELWYN, 1957). PORCELLATI (1958) has stated that phosphorylserine is partly co-precipitated when barium hydroxide is used to remove orthophosphate from brain extracts but chromatograms of the barium precipitate (treated with Amberlite IR-120 (NH^+_4) failed to reveal any substance other than orthophosphate. Amounts of phosphorylserine equivalent to 2–8 μg P run on paper chromatograms could be recovered to the extent of 80–95 per cent using ethanol-NH_3 and phenol-NH_3. 'Norit' A appeared to adsorb about 20 per cent of phosphorylserine when small amounts (*ca.* 20 μg P) were added to a suspension of 200 mg of the charcoal in 10 per cent TCA. (This compares with 0 per cent for phosphorylethanolamine, 12 per cent for glycerophosphate and <25 per cent for phosphorylcholine.)

The uptake of [^{32}P]-*orthophosphate into phospholipids and phosphate esters.* In Table 1 are given the values for the uptake of [^{32}P]-orthophosphate into various fractions. The uptake into phospholipid fractions is comparable with that previously recorded (ANSELL and DOHMEN, 1957) and that into phosphorylethanolamine is comparable with the values for 3 hr. given by ANSELL and DAWSON (1951) in which the uptake was measured as the ratio specific radioactivity phosphorylethanolamine P : specific radioactivity inorganic + acid-labile P. It is noteworthy that the formation of phosphorylcholine in the three-hour period is twice as fast as the corresponding ethanolamine ester and the specific radioactivity is much higher than the acid-soluble phosphate as a whole.† This indicates an initial active transfer of phosphate via adenosine triphosphate (ATP)

* Recent work by BAKER and PORCELLATI has shown that the cerebral content of phosphorylserine is only about a third of this value (R. W. R. BAKER personal communication).

† TKESHELOSHVILI (1956) *Trud. Inst. Fiziol. Akad. Nauk. Gruz. S.S.R.* **10**, 317 has measured the uptake of [^{32}P]-orthophosphate into cerebral phosphorylethanolamine and phosphorylcholine (seen in abstract only. *Int. Abstr. biol. Sci.* (1959) **12** Abstr. No. 1833).

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and choline phosphokinase (Fig. 2) but the radioactivity in phosphorylcholine did not fall very rapidly. DAWSON (1956) has shown that there is a rapid incorporation of labelled orthophosphate into liver phosphorylcholine but in that tissue the radioactivity of the ester is rapidly lost. Presumably phosphorylcholine is not lost from brain tissue very easily owing to the operation of the blood-brain barrier.

TABLE 1.—THE UPTAKE OF [^{32}P]-LABELLED ORTHOPHOSPHATE INTO LIPID PHOSPHATE ESTERS AND PHOSPHOLIPID FRACTIONS OF RAT BRAIN AFTER 3 HR

Phosphorus fraction	Ratio $\frac{\text{Specific radioactivity fraction}}{\text{Specific radioactivity acid-soluble P}} \times 1000$
Phosphorylcholine	1754 ± 300 (6)
Phosphorylethanolamine	788 ± 29 (6)
Diphosphoinositide	564 ± 101 (3)
Phosphatidyl choline	44·2 ± 3·4 (6)
Total phospholipid	37·8 ± 1·5 (6)
Phosphatidy(a)l ethanolamine	23·7 ± 4·3 (6)
Phosphatidyl serine	15·6 ± 4·0 (3)

The values are given ± SD with the numbers of animals in brackets.

The effect of insulin. It can be seen from Table 2 that the insulin significantly depressed the synthesis of brain phosphorylethanolamine by 17 per cent but not significantly that of phosphorylcholine. In previous experiments (ANSELL and DOHMEN, 1957) it was shown that there was a significant depression of phosphatidyl ethanolamine and phosphatidylcholine synthesis and this is confirmed in the results shown in Table 2.

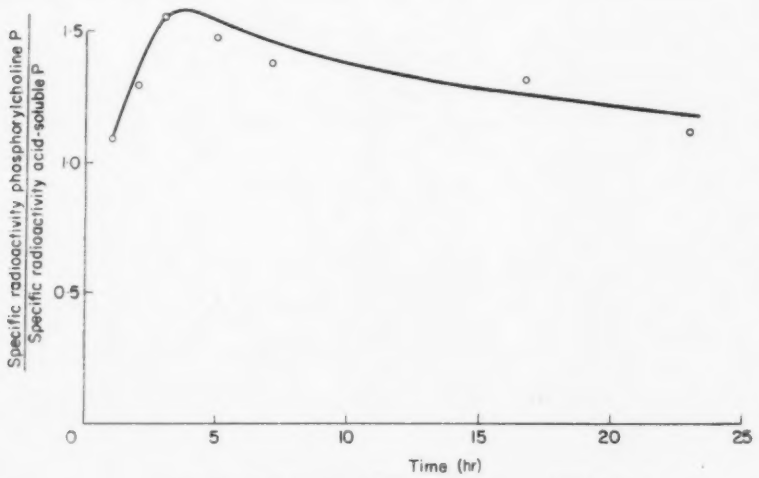

FIG. 2.—The uptake of ^{32}P into brain phosphorylcholine.

DAWSON (1956) has noted a rise in the amount of phosphorylcholine in liver after the injection of insulin. Normal values for brain phosphorylcholine in the present series of experiments were 24 ± 9·4 (8) for the normal animals and 29·9 ± 6·7 (12) μg/g brain for those in insulin hypoglycaemia which perhaps indicates a slight

increase but the recoveries were low. Values for phosphorylethanolamine of 127 ± 14 (6) μg phosphorylethanolamine/g brain were obtained for the normal series and 121 ± 9 (6) for the hypoglycaemic animals.

It is apparent that in insulin hypoglycaemia the rate of synthesis of phosphorylethanolamine is slowed down but that the brain levels remain normal. The 45 per cent decrease in uptake of labelled phosphate into phosphatidyl ethanolamine was nearly three times as great as the decrease for phosphorylethanolamine, however, so that there is presumably a point intermediate between the phosphorylation of ethanolamine

TABLE 2.—THE EFFECT OF INSULIN ON THE UPTAKE OF [^{32}P]-LABELLED ORTHOPHOSPHATE INTO CEREBRAL PHOSPHATE ESTERS AND PHOSPHOLIPIDS AFTER A 3 HR EXCHANGE PERIOD

Phosphorus fraction	Ratio $\frac{\text{Specific radioactivity fraction}}{\text{Specific radioactivity acid-soluble P}} \times 1000$		Probability (P)*
	Normal	Insulin	
Phosphorylcholine	1786 ± 507 (9)	1622 ± 311 (9)	0·3–0.4
Phosphorylethanolamine	799 ± 123 (9)	666 ± 120 (9)	0·01–0·001
Total phospholipid	40·0 ± 2·8 (10)	26·5 ± 7·1 (11)	<0·001
Phosphatidyl choline	56·4 ± 6·5 (6)	39·5 ± 10·8 (5)	0·02–0·01
Phosphatidy(a)l ethanolamine	29·1 ± 1·5 (6)	19·0 ± 4·0 (5)	<0.001

The experiments were carried out on three groups of litter mates. Half of each group were control animals which had been fasted for the same length of time. The values are given ±SD and the numbers in brackets refer to the number of animals used.

* t-test.

and the incorporation of the phosphorylated base into the complete phospholipid molecule which is also susceptible to insulin hypoglycaemia.

Insulin hypoglycaemia is known to lower the ATP level of cerebral tissues to a great extent (OLSEN and KLEIN, 1947) which might be reflected in a slower formation of phosphorylethanolamine and phosphorylcholine. Choline phosphokinase and the enzyme responsible for ethanolamine phosphorylation are known to be ATP-dependent (WITTENBERG and KORNBERG, 1953; MCMURRAY, STRICKLAND, BERRY and ROSSITER, 1957). The fact that the phosphorylation of choline is scarcely reduced in insulin hypoglycaemia whereas that of ethanolamine is significantly decreased might indicate that the action of insulin is not on the energy supply in this instance. It is possible, however, that the ATP level necessary for maintaining a normal rate of phosphorylcholine formation is much lower than that required for the corresponding ethanolamine ester. Alternatively the free choline concentration might rise and compensate for the smaller amount of available ATP.

In thiopentone anaesthesia the depression in phosphatidyl ethanolamine synthesis is confined to a step or steps later than the phosphorylation of ethanolamine (ANSELL and MORGAN, 1958); in the two conditions therefore the effects on phosphatidyl ethanolamine metabolism are different. It is not known from the result of the present investigation whether or not there is any difference between the action of insulin and thiopentone on phosphatidyl choline metabolism, although in barbiturate anaesthesia

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the cerebral ATP level is raised (STONE, 1940). Further studies on other intermediates e.g. cytidine diphosphate choline would no doubt throw further light on this problem.

SUMMARY

(1) A method adequate for the determination of the specific radioactivities of phosphorylcholine and phosphorylethanolamine in brain is described.

(2) Levels of phosphorylcholine and phosphorylethanolamine in whole rat brain tissue have been measured and the results compare with those of other workers. Phosphorylserine was not observed on chromatograms of extracts prepared as described.

(3) The uptake of labelled orthophosphate into brain phosphorylcholine *in vivo* after a short exchange period was approximately twice that into phosphorylethanolamine and this was paralleled by the uptake into the corresponding phospholipids.

(4) In insulin hypoglycaemia there was a significant depression of the uptake of labelled orthophosphate into phosphorylethanolamine but not into phosphorylcholine. The uptake of labelled phosphate into both phosphatidyl choline and ethanolamine was significantly depressed.

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CHANGES INDUCED BY PROLONGED ADMINISTRATION OF NICOTINE IN THE DISTRIBUTIONS OF CHOLINESTERASES AND ACID PHOSPHATASE IN THE ADRENAL MEDULLA OF THE RAT

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PROLONGED administration of nicotine causes a pronounced hyperplasia of the adrenal medulla in the rat, as was demonstrated by STAEMMLER (1935) over 20 years ago. ERÄNKÖ (1955*a*) observed that this hyperplasia is associated with about a five-fold increase in the noradrenaline content of the adrenal without any significant change in the adrenaline content. Moreover, the volume of the noradrenaline-containing medullary parenchyma, as demonstrated histochemically by formalin-induced fluorescence (ERÄNKÖ, 1951, 1952, 1955*a*,*b*), increased in proportion to the noradrenaline content, while there was no significant change in the volume of the adrenaline-containing medullary tissue.

Earlier work has indicated that the activities of enzymes such as acid phosphatase (APh) (ERÄNKÖ, 1951, 1952) and cholinesterases (ALLEN, ERÄNKÖ and HUNTER, 1958; ERÄNKÖ, 1958, 1959) are different in the regions of the two types of adrenomedullary cells of the rat. It seemed therefore to be of interest to investigate histochemically these enzymes in the hyperplastic adrenal medulla of nicotine-injected rats. The present study shows that nicotine treatment brings about marked changes in the distributions and activities of these enzymes.

MATERIALS AND METHODS

Experimental. Young male albino rats with a mean weight of about 70 gm were daily injected subcutaneously with nicotine, Sundays excepted. Nicotine (Nicotin puriss., Dr. Theodor Schuchart G.m.b.H., Görlitz, Germany) was diluted with distilled water to a concentration of 2 mg/ml. The number of injected rats was initially 60, and the daily dose was 1 mg/rat. Because of high mortality, the daily dose was reduced after 9 days to 0·4 mg/rat. This dose was maintained for about 2 months. Thereafter 0·5 mg was given daily until the end of the treatment, which lasted about 9 months. Only 16 rats survived, and their mean weight was about 200 gm at the end of the treatment.

Control rats, reared under conditions identical with those of the experimental animals but left uninjected, did not show a weight gain significantly different from that of the nicotine-injected rats.

The injected rats were killed by decapitation 1–4 days after the last injection. The variable length of the period between the last injection and killing did not affect the results. Each time any of the injected animals were killed, a number of controls were also decapitated.

Histochemical methods. Adrenals were fixed for 2–4 hr in a mixture containing 1 vol. of neutral analytical 35% (w/w) formaldehyde solution, 6 vol. of 2% (w/v) calcium chloride and 3 vol. of distilled water. They were sectioned with a freezing microtome at 20–50 μ, rinsed in distilled water, floated on clean slides, and allowed to dry on them.

Unstained dry sections were examined in the fluorescence microscope to register the fluorescent medullary cell islets, which are presumably composed of the noradrenaline-containing secretory cells (ERÄNKÖ, 1955*b*). After fluorescence photomicrography the same sections could be used for the

demonstration of cholinesterases of APh, whose distribution can be registered by ordinary photomicrography.

Cholinesterases were demonstrated using the improved method described by KOELLE (1951). However, to differentiate between acetylcholinesterase (AChE) and non-specific cholinesterase (nsChE), selective inhibitors were used, i.e. 1:5-*bis*-(4-trimethylammonium-phenyl)pentan-3-one di-iodide (62.C.47) (BURGEN, 1949), which is a specific inhibitor for acetylcholinesterase, and tetra-*iso*propylpyrophosphoramide (*iso*-OMPA) (ALDRIDGE, 1953), which is a selective inhibitor of non-specific cholinesterase (see PEPLER and PEARSE, 1957; ERÄNKÖ, 1958, 1959). A reaction obtained with acetylthiocholine (AThCh) as a substrate in the presence of 10^{-6} M-*iso*-OMPA was ascribed to AChE, and that obtained with butyrylthiocholine (BThCh) and 10^{-5} M-62.C.47 to non-specific cholinesterase. Sections incubated with both substrates and with both of the above-mentioned inhibitors or 10^{-5} M-eserine served as controls. Before incubation in the substrate mixtures the sections were in all instances pre-incubated in a similar but substrate-free mixture containing either or both inhibitors. Details of the technique and results obtained with normal rat adrenals are described elsewhere (ERÄNKÖ, 1958, 1959).

For the demonstration of APh a modification of GOMORI's (1941) glycerophosphate—lead nitrate method was used (ERÄNKÖ, 1952).

RESULTS

Fluorescent cell islets

In agreement with the results obtained earlier (ERÄNKÖ, 1955*a*) it was found that the medullary volume and the relative amount of fluorescent medullary tissue were greatly increased in the nicotine-injected rats (Figs. 1, 2, 7, 8, 15 and 16). The increase was due to enlargement and fusion of the individual fluorescent islets rather than to increase in their number. However, the hyperplastic medullary tissue had less tendency to form rounded nodules bulging into the cortex than in the earlier work (ERÄNKÖ, 1955*a*). Presumably this difference is due to the smaller dosage of nicotine in the present study.

Cholinesterases

The histochemical reaction observed in sections of the adrenals from control and nicotine-treated rats was always absent when both types of cholinesterase were inhibited either by eserine or by a combination of 62.C.47 and *iso*-OMPA (Figs. 13 and 14). This proves that only cholinesterases are involved in the histochemical reaction.

Acetylcholinesterase. In the adrenals of the control rats, a strong AChE reaction was found in the coarse nerve trunks, in the ganglion cells, and in the fine fibres which formed a fine network over the whole medulla and which showed small ovoid, strongly positive bodies along their lengths (Fig. 3). The regions of the medullary areas corresponding to the fluorescent cell islets exhibited a stronger reaction, presumably because of the denser network of fibres around the noradrenaline-containing cells (Figs. 1, 3 and 5).

In the adrenals of the nicotine-injected rats the distribution of AChE was in many respects similar. Thus, coarse and fine nerve fibres and the ovoid bodies along the latter fibres were strongly positive. However, there were many regions in the medulla, particularly near the corticomedullary junction, where the medullary parenchyma was entirely negative (Figs. 4 and 6). These negative areas were found to correspond to the regions of the fluorescent islets, and they probably represent newly formed medullary tissue which, although the cells are capable of forming noradrenaline, is devoid of fibres with AChE activity (Figs. 2 and 4). Whether or not nerve fibres

EXPLANATIONS OF THE FIGURE PLATES

The figures have been arranged in pairs so that photomicrographs of the adrenals of the controls are on the left, those of the nicotine-treated rats on the right.

There are 3 such pairs of fluorescence photomicrographs, i.e., Figs. 1 and 2, 7 and 8, 15 and 16. In these figures the blood vessels are black, the main medullary parenchyma dark grey, the cortical tissue, present in the upper parts of Figs. 1, 2, 7, 8 and 15, light grey, and the fluorescent medullary islets white.

Below each fluorescence photomicrograph is another photomicrograph taken in transmitted light of the same section after histochemical demonstration of AChE, nsChE or APh. In these figures the blood vessels, which serve as useful land-marks when comparing the two photomicrographs of the same section, are white, regions devoid of enzyme activity grey, enzymically active regions dark grey or black. Owing to light refraction, cortical tissue, though essentially devoid of demonstrable enzyme activity, appears darker than the enzymically inactive regions of the medulla.

To facilitate the comparison of the corresponding regions in the fluorescence photomicrographs and those taken after histochemical demonstration of the enzymes, one region of fluorescent cells is indicated in each figure by two arrow-heads.

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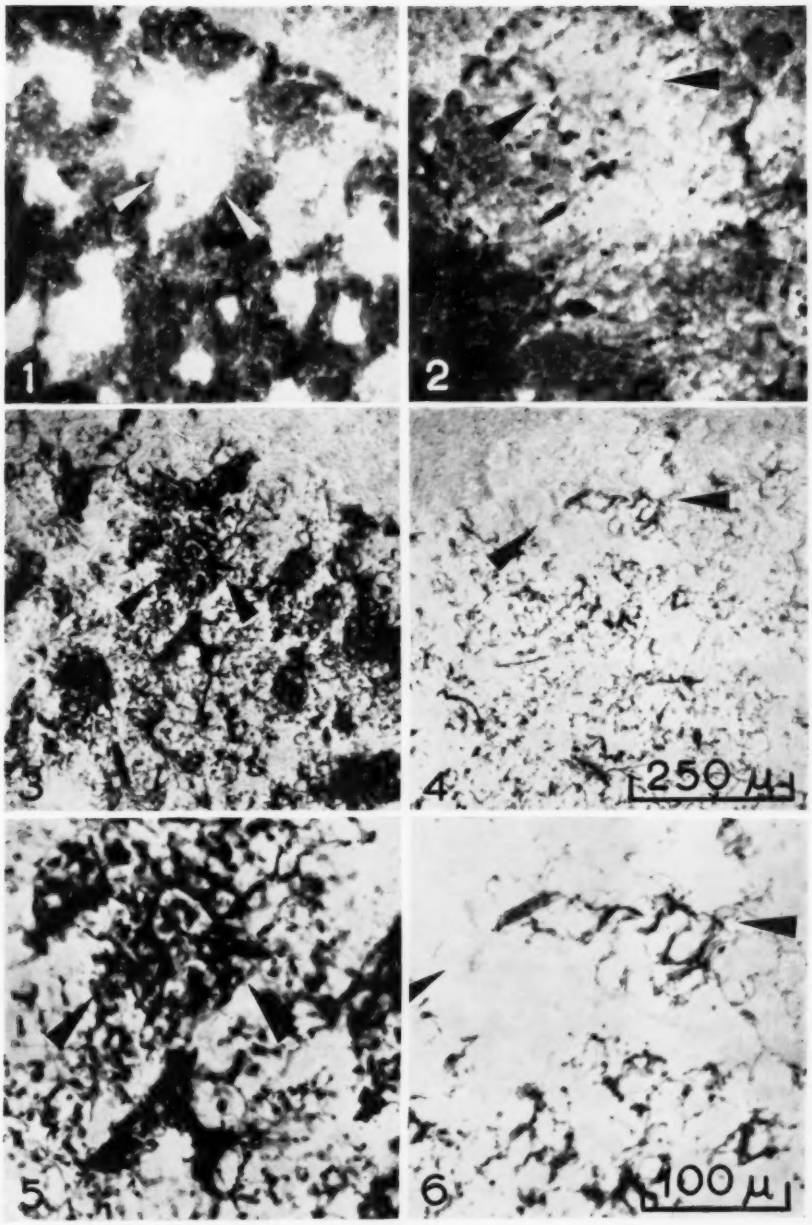

PLATE 1

FIG. 1. Fluorescence, control rat. ×90.
FIG. 2. Fluorescence, nicotine rat. ×90.
FIG. 3. Same field as in Fig. 1. AChE. ×90.
FIG. 4. Same field as in Fig. 2. AChE. ×90.
FIG. 5. Detail of Fig. 3. ×200.
FIG. 6. Detail of Fig. 4. ×200.

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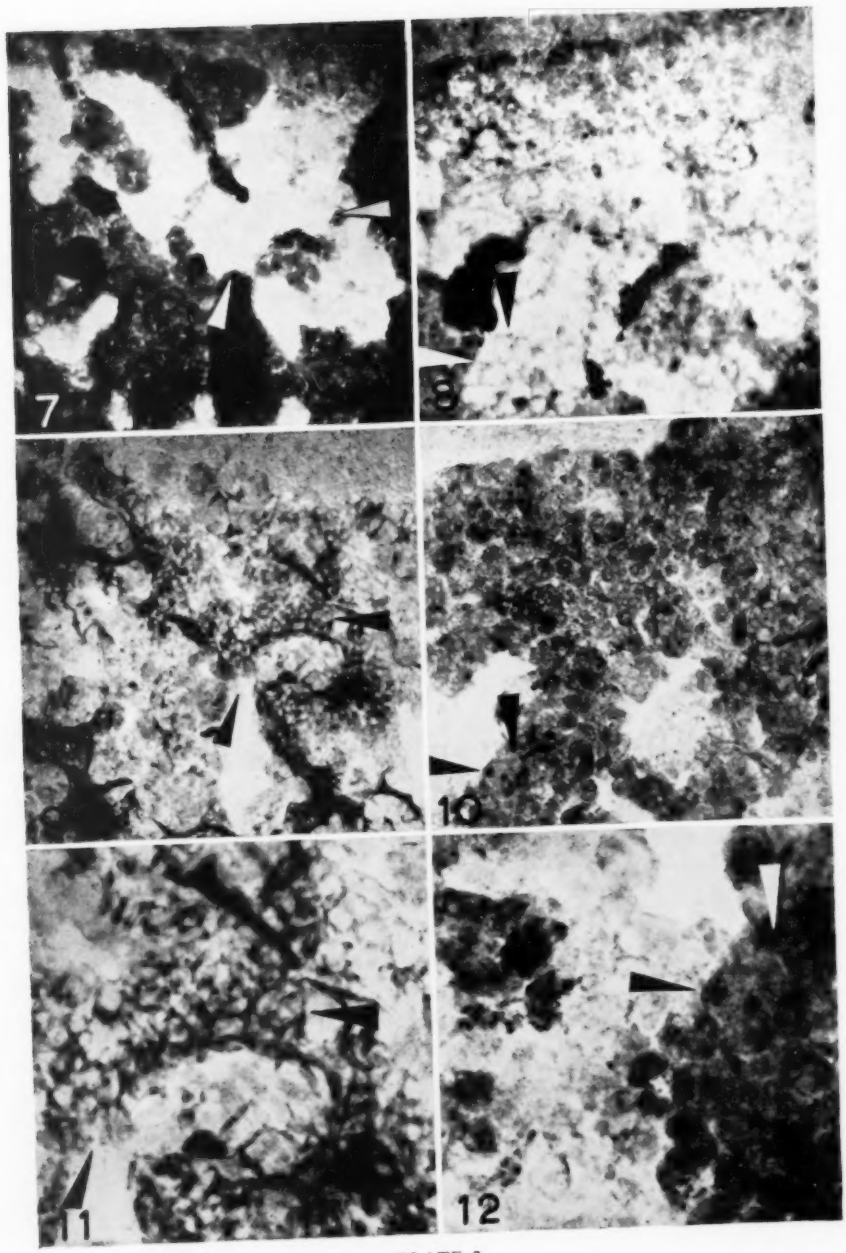

PLATE 2

FIG. 7. Fluorescence, control rat. ×90.
FIG. 8. Fluorescence, nicotine rat. ×90.
FIG. 9. Same field as in Fig. 7. nsChE. ×90.
FIG. 10. Same field as in Fig. 8. nsChE. ×90.
FIG. 11. Detail of Fig. 9. ×200.
FIG. 12. Detail of Fig. 10. ×200.

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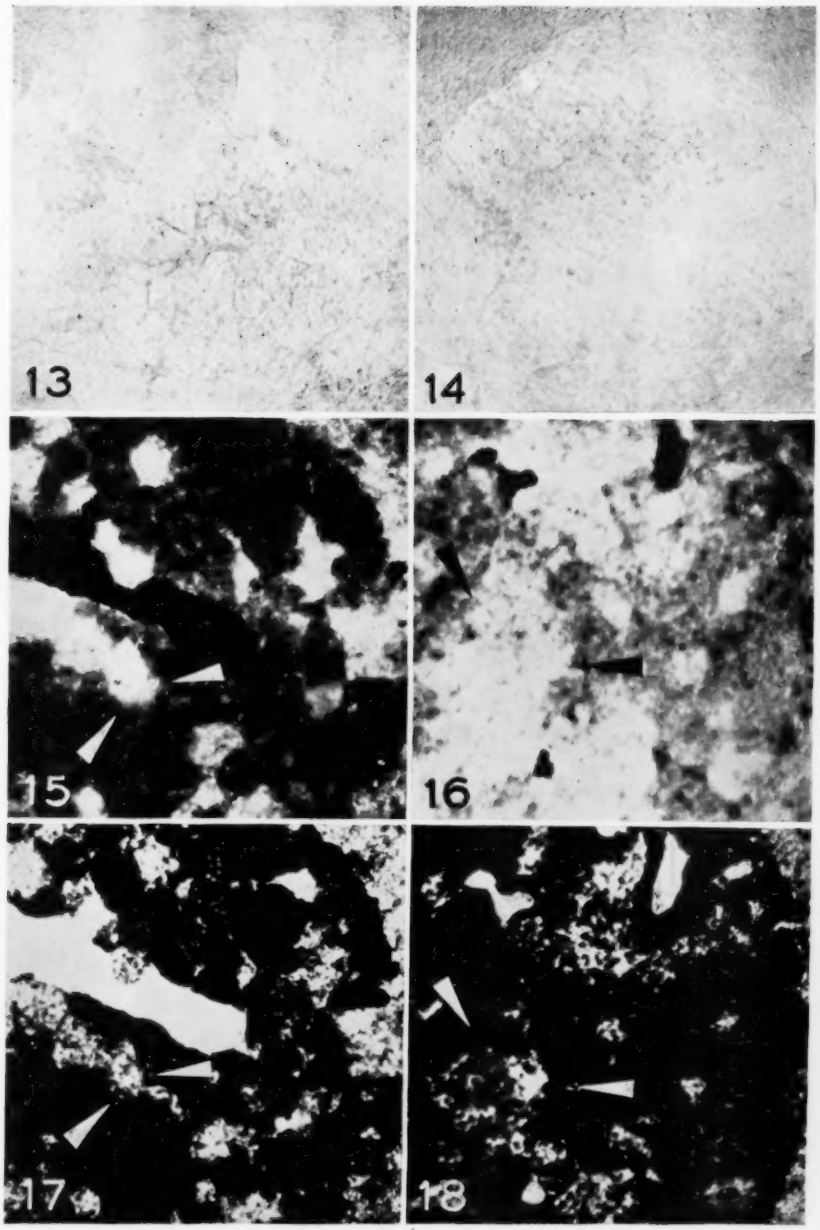

PLATE 3

FIG. 13. AThCh + BThCh + 62.C.37 + *iso*-OMPA. Control rat. ×90.

FIG. 14. AThCh + BThCh + 62.C.47 + *iso*-OMPA. Nicotine rat. ×90.

FIG. 15. Fluorescence, control rat. ×90.

FIG. 16. Fluorescence, nicotine rat. ×90.

FIG. 17. Same field as in Fig. 15. APh. ×90.

FIG. 18. Same field as in Fig. 16. APh. ×90.

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were really lacking from these regions, cannot be finally decided with the methods used in the present study. In any case, such a negative reaction in the region of fluorescent medullary tissue is the more remarkable, since the positive network in similar regions of normal adrenals is even somewhat denser than in the regions of the non-fluorescent medullary cells.

Non-specific cholinesterase. In the normal rats the medullary reaction was positive in the coarse nerve fibres and the small medullary ganglia, as well as in the dense network of fibres entangling the fluorescent parenchymal cell islets (Figs. 7, 9 and 11). The remaining medullary parenchyma, which is composed of the non-fluorescent, presumably adrenaline-containing cells, and of the fine nerve fibres around these cells, reacted but weakly.

The distribution pattern of nsChE was different in the adrenal medulla of the nicotine-treated animals. There were positive coarse fibres and nerve trunks both in the regions of the cortex and the medulla but in the medulla they were overshadowed by large groups of cells with a strongly positive reaction (Fig. 10). Comparison of fluorescence photomicrographs (Fig. 8) with those taken of the same area after demonstration of nsChE (Fig. 10) showed that the fluorescent cell islets regularly exhibited a strong enzyme activity. At higher magnification it was seen that the enzyme activity, which in normal adrenals was fairly sharply restricted to the fibre network surrounding the noradrenaline-containing cells (Fig. 11), was uniformly positive in the cytoplasm of the medullary parenchymal cells of the nicotine-injected rats. In addition, a strongly positive reaction was seen in small spherical bodies between the cells, while no fibre network at all was visible (Fig. 12).

Acid phosphatase

As in earlier studies (ERÄNKÖ, 1952), APh reaction was selectively detected in the non-fluorescent areas of the medulla of the control rats, the fluorescent cell islets remaining essentially negative (Figs. 15 and 17). On the other hand, the region of the larger islets formed by the fluorescent medullary cells in the adrenals of the nicotine-injected rats gave a positive APh reaction (Figs. 16 and 18). Closer examination showed that the centre of these islets, which tended to fluoresce brighter than the periphery, showed as a rule a weaker APh reaction than the rest of the medulla (Fig. 18).

DISCUSSION

The present study confirms the earlier observation that nicotine causes an increase in the volume of the parenchyma formed by those medullary cells which fluoresce after treatment with calcium-formol, i.e. the noradrenaline-containing ones, (ERÄNKÖ, 1955*a*). It also shows that alterations in the activities of the three enzymes now investigated are restricted to the regions of the fluorescent cells.

It is of interest to note that opposite effects were caused by treatment with nicotine in the APh activity and in the AChE activity. While the former increased, the latter decreased. These changes did not occur throughout the fluorescent parenchyma; they were restricted to only parts of it, mainly to the peripheral areas of the large fluorescent islets, where the formation of new noradrenaline-containing cells had most likely occurred. The centre of these islets, probably representing 'old' fluorescent cells, showed no, or less, changes in the APh and AChE activities.

One might speculate that the absence of AChE activity from these regions is simply due to the formation of new parenchymal tissue without corresponding formation of nerve fibres. Similarly, the strong APh activity could be taken as a sign of the primitive nature of the newly formed cells. However, it was shown that a single injection of 0·25 mg of reserpine causes a complete disappearance of noradrenaline in 24 hr, as demonstrated histochemically with the iodate reaction (HILLARP and HÖKFELT, 1955), from the noradrenaline-containing hyperplastic medullary cell islets of nicotine-treated rats (unpublished). Since similar reserpine-induced depletion is abolished in normal rats by splanchnic denervation (ERÄNKÖ and HOPSU, 1958), it would seem that the hyperplastic noradrenaline cell islets in the adrenals of the nicotine-injected rats are capable of responding to nervous stimuli by increased secretion of noradrenaline.

In contrast to the changes in the activities of APh and AChE, which were limited to a part of the fluorescent cell islets, nicotine treatment caused the appearance of nsChE throughout the fluorescent islets, which in the adrenals of the control rats exhibited little activity of nsChE. Thus, not only the newly formed noradrenaline-producing cells were affected, but also those noradrenaline-containing cells which were present before nicotine administration. The significance of the changes in nsChE are at present obscure, but it is of interest to note that denervation of normal adrenals is not accompanied by a loss of nsChE from the networks around the fluorescent cells, in spite of a complete disappearance of histochemically demonstrable AChE from the medullary nerve net (ERÄNKÖ, 1958; ERÄNKÖ, HOPSU and RÄISÄNEN, 1959).

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SUMMARY

Hyperplasia of the medulla was induced in male albino rats by daily subcutaneous injections of nicotine for 9 months. The newly formed medullary tissue which exhibited a strong fluorescence in ultraviolet light after fixation in calcium-formol differed histochemically from the fluorescent medullary tissue in normal rat adrenals. Fibres exhibiting a positive reaction for acetylcholinesterase were fewer or lacking in the newly formed medullary parenchyma of nicotine-treated rats. The adrenals of these animals, on the other hand, showed a strong activity of non-specific cholinesterase in the cytoplasm of all fluorescent medullary cells and a strongly positive acid phosphatase reaction in the peripheral parts of the fluorescent islets which in normal adrenals give essentially negative reactions for both enzymes.

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OBSERVATIONS ON ENDOGENOUS METABOLISM IN BRAIN *IN VITRO* AND *IN VIVO**

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IT has been shown that nervous tissue can utilize substances other than glucose (GERARD, 1932). Using the isolated cat brain perfused with artificial glucose-free blood, it was shown that oxidation of endogenous glucose, lactic acid and glycogen accounted for only about 20 per cent of the oxygen uptake (GEIGER, MAGNES and GEIGER, 1952). With guinea pig brain cortex slices, incubated without glucose, the endogenous glycogen, lactic acid and glucose account at most for 35 per cent of the oxygen uptake (TAKAGAKI, HIRANO and TSUKADA, 1957). Electrical stimulation of the superior cervical ganglion of the rat increases the oxygen consumption independently of the exogenous glucose utilization (LARRABEE, HOROWICZ, STEKIEL and DOLIVO, 1957). The carbon dioxide production by the cat brain during metrazol convulsions and electrical shock cannot be accounted for by the utilization of endogenous glucose (ALLWEISS and MAGNES, 1958).

The utilization of unknown endogenous substrates by nervous tissue *in vitro* is associated with increased ammonia formation (WEIL–MALHERBE, 1952; TAKAGAKI *et al.*, 1957). Increase of ammonia formation in the brain following stimulation has also been demonstrated *in vivo* (for reviews see VRBA, 1957*b*; VRBA, FOLBERGROVÁ and KANTŮREK, 1958). The concentration of ammonia in the brain may increase *in vivo* up to 25–40 mg/100 g fresh brain during preconvulsive and convulsive stages of high oxygen tension intoxication (GERSHENOVITSCH and KRITSHEVSKAYA, 1954).

The brain contains adenylic acid deaminase, ATP-deaminase, glutamic acid dehydrogenase, glutaminase, hexosamine deaminase, monoamine oxidase and their corresponding substrates. It was demonstrated that these enzymes are not responsible for ammonia formation and it has been suggested that ammonia formation in brain slices incubated without additional substrates may result from proteolytic processes (WEIL–MALHERBE and DRYSDALE, 1957; WEIL–MALHERBE and GREEN, 1955). We have recently observed that the accumulation of non-protein nitrogen exclusive of ammonia (NPN) in brain cortex slices is not commensurate with ammonia formation, and that ammonia formation is inhibited by the presence of potassium ions without a parallel inhibition of NPN formation (VRBA *et al.*, 1958). Therefore putrifactive proteolytic processes as a direct cause of the accumulation of ammonia does not seem likely.

The purposes of the present investigation were: (a) to attempt to elucidate the source of the ammonia formed from endogenous substrates during incubation of nervous tissue; (b) to provide additional information on the inhibitory effect of

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potassium on this process; (c) to describe some 'non-glucose substrates' utilized; and (d) to compare the processes occurring *in vivo* and *in vitro*. A preliminary report on this work has been published elsewhere (VRBA and FOLBERGROVÁ, 1958*b*).

METHODS

(a) *Experiments* in vitro

Experimental design. The nitrogen metabolism of guinea pig brain cortex slices was studied. Since it is difficult to remove adhering liquid in a reproducible fashion, calculations based on wet weight of tissue slices may be erroneous. To diminish the effect of variation between slices, batches of slices weighing up to 400–600 mg wet weight of slices were used for each analysis and the results were expressed as percentage of total nitrogen disregarding wet weight.

Each experiment was performed with twelve Warburg vessels containing 4 ml of Krebs–Ringer-phosphate-saline without substrate. To six vessels serving as non-incubated controls 1 ml of 50% (w/v) trichloroacetic acid (TCA) was added before introducing the slices. Fresh guinea pig brain cortex slices (60-100 mg wet weight) were quickly weighed to the nearest 0·1 mg and introduced into all twelve vessels which were kept in an ice bath. Six vessels not containing TCA were incubated at 37° in oxygen for 5 hr with shaking, after which period the vessels were detached from the manometers and 1 ml of 50% (w/v) TCA was added to each incubated vessel and their contents were combined. The non-incubated vessels were similarly treated. TCA extracts were prepared from both samples as rapidly as possible.

The estimation of free ammonia and non-protein nitrogen. Slices, suspension medium and TCA extracts of fresh and incubated slices were prepared according to VRBA *et al.*, (1958). The final volume of the TCA-extract was 25 or 50 ml (10% w/v TCA). This extract was kept in a water bath for 75 min at 70° and then made up to the original volume with 10% TCA (RICHTER and DAWSON, 1948). Ammonia was then estimated in the extract as previously (VRBA *et al.*, 1958). The value obtained represents the sum of free and bound TCA-soluble ammonia. The bound TCA-soluble ammonia is derived from glutamine. It should be noted that the TCA-soluble glutamine in brain cortex slices represents only a small fraction of the total TCA-soluble ammonia (WEIL-MALHERBE and GREEN, 1955). A 5 ml aliquot of the TCA-extract was digested and then made up to 12 ml with water and assayed for ammonia according to MARKHAM (1942).

Estimation of 'lipid nitrogen'. The remaining tissue was extracted with 8 ml acetone, then 5 ml ethanol–ethyl ether (3 : 1 v/v) and 5 ml of a chloroform–methanol mixture (2 : 1 v/v): finally the residue was extracted with 5 ml of ethyl ether. Details were described previously (VRBA *et al.*, 1958). The combined extracts were made up to 25 ml with methanol and a 5 ml aliquot was evaporated on the steam bath. To the dry residue 2 ml of the mixture of SOBEL, MAYER and GOTTFRIED (1944) was added and they were digested at approximately 300°: 0·1 ml of 30% H_2O_2 was added occasionally during digestion until full decolorization occurred. The mixture was then made up with water to 12 ml and ammonia was estimated in a 5 ml aliquot, using a MARKHAM (1942) apparatus. The distillate (12–14 ml) was collected and the ammonia was trapped in 1 ml of 0·1 N-HCl. The distillate was made up to 15 ml with water, 2 ml of Nessler reagent were added and readings were taken at 490 mμ.

Estimation of 'nucleic acid nitrogen'. The tissue remaining after 'lipid nitrogen' extraction was further extracted with 20 ml 10% (w/v) NaCl solution in a boiling water bath for 2 hr. After separation of the supernatant by centrifugation (about 1000 g) the remaining tissue was extracted five times for 1 hr with 6 ml of the NaCl solution. The combined supernatants, representing the nucleic acids and other nitrogenous compounds (DAVIDSON and SMELLIE, 1952), were made up to 50 ml with 10% NaCl. Of this, 5 ml aliquots were digested and ammonia was estimated as described.

Estimation of 'protein nitrogen'. The extracted tissue was hydrolysed with 6 ml of 20% (w/v) HCl and the precipitated humin was separated by centrifugation and washed several times with water. The combined supernatants were made up to 100 ml, and 20 ml aliquots were digested and made up to 12 ml with water: 8 ml were distilled in the Markham apparatus, the ammonia was collected in 10 ml of a 2% (w/v) boric acid solution, containing MA and Zuazaga's indicator. The distillate (15 ml) was collected and ammonia was titrated with 0·01 N-H_2SO_4. Ammonium sulphate standards were included. The undissolved humin residues were digested with 2 ml of the digestion mixture and made up to 12 ml. Ammonia was estimated in triplicate on 2 ml aliquots after distillation and nesslerization.

When the ammonia concentration in the distillate ranged from 0·01 to 0·1 mg nesslerization was used. The higher concentrations in the protein digests were titrated with 0·01 N-H_2SO_4. Triplicate analyses were performed where possible. The protein residue was hydrolysed and the supernatant assayed in triplicate. The remaining humin residue was assayed after separate digestion. The alternative procedure of drying the protein residue and weighing out three portions for digestion was avoided, in view of the fact that the drying process itself could lead to loss of ammonia and the hygroscopic properties of proteins make the accuracy of a weighing debatable.

Spectrophotometrical estimation of nucleic acids. The optical density of the NaCl-extract was measured in ultraviolet light using the Soviet spectrophotometer SF–4, and absorption curves were constructed. The measured densities were referred to 10 mg total nitrogen of slices.

TCA-soluble and TCA-insoluble orcinol-positive substances (*pentoses*) were determined by the orcinol-method of MEJBAUM (1939). As bound pentoses may show a different optical density with this method in comparison with free pentoses (MEJBAUM, 1945), no standards were used and the densities of extracts of fresh slices were compared (after correction to the standard weight of 10 mg total nitrogen of slices) with similarly calculated densities of the extracts of incubated slices.

In cases where the final volume of the TCA extract was 50 ml, 3 ml of the extract was mixed with 3 ml of the orcinol reagent (1% orcinol and 0·5% $FeCl_3$ in concentrated HCl) and kept in a boiling water bath for 20 min. The optical density was measured at 660 mμ using a similarly treated TCA-solution as blank . Where the final volume of TCA-extract was only 25 ml, 1 ml of the TCA-extract was diluted with 2 ml of water and 3 ml of orcinol-reagent added. For the estimation of TCA-insoluble pentoses, 3 ml of the NaCl-extract was similarly treated as described above.

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(b) *Experiments* in vivo

Compulsory swimming was used to stimulate the rat brains. Two experiments were performed (A and B), each with 24 rats. These were divided into four groups of three rats each to serve as controls: four similar groups were forced to swim for 4·5 or 6·5 hr. Both groups were decapitated and the heads dropped into liquid air. The frozen brains were ground to powder three at a time. Of the total powder from each group 1–1·5 g was put into weighed centrifuge tubes containing 8 ml of 10% (w/v) TCA. The sample weight was determined by differential weighing. A trichloroacetic acid extract was then prepared as described previously (VRBA, 1956). The final volume of the extract was 25 ml (10% w/v TCA), and the extract was hydrolysed as in the *in vitro* experiments (see methods, Section (a)). The sum of the free and bound TCA-soluble ammonia was determined in three 2 ml aliquots of the extract. It should be noted that this value in extracts of frozen brains represents mainly TCA-soluble glutamine, and only negligible amounts of ammonia (RICHTER and DAWSON, 1948; VRBA, 1954*b*). The rest of the tissue was then fractionated and analysed as described in Section (a), with the following modifications. The TCA-extract (2 ml) was digested, made up to 12 ml and ammonia was estimated in a 4 ml aliquot. The lipids were extracted with 10 ml of each solvent and the combined supernatants were made up to 50 ml with methanol. A 5 ml aliquot was digested after evaporation, the digest was made up to 12 ml and ammonia was estimated in 2 ml aliquots. The final volume of the NaCl-extract was 100 ml, of which 20 ml were digested and the digest made up to 20 ml. Ammonia was then estimated in 2 ml aliquots by nesslerization. The volume of the protein hydrolysate was 100 ml and 20 ml were digested: the digest was made up with water to 20 ml, and ammonia was estimated in 5 ml aliquots by titration. The digest of the humins was made up to 20 ml and ammonia was estimated in 1 ml aliquots.

These modifications were necessary to keep the amount of ammonia in the aliquots within the optimal analytical ranges, since more material was used in the studies *in vivo*. The results are given per gram fresh weight, because the quantity of nitrogenous substances removed by the blood is unknown. Experiment A employed twenty-four rats weighing 150–200 g. Marked exhaustion was produced by enforced swimming in a water bath at 30–33°, 40 × 60 cm in area and 40 cm deep for 4·5 hr. Experiment B employed twenty-four male rats 67 days old weighing approximately 200 g each. They were specially bred and animals showing growth abnormalities were excluded. Thus a more homogeneous group of animals was obtained. These rats were forced to swim 6·5 hr and the depth of the bath was decreased to 25 cm. These animals showed less fatigue than the previous groups. Possibly the degree of exhaustion after swimming is influenced by the depth of the water and the general physiological conditions. In both experimental groups all the rats were forced to swim simultaneously. The crowded conditions evoked intensive muscular activity.

TABLE 1.—NITROGEN CHANGES IN TCA-SOLUBLE, 'LIPID', 'NUCLEIC ACID' and' PROTEIN' FRACTIONS OF GUINEA PIG BRAIN CORTEX SLICES DURING AN INCUBATION OF 5 HR IN THE ABSENCE OF ADDED SUBSTRATE

The figures are obtained by analysis of a combined batch of slices from six Warburg vessels, each vessel containing 60–100 mg fresh weight of slices.

Fraction			Experiment 1					
			Initial		Final		Difference	
			μg N	N (% of total N)	μg N	N (% of total N)	Change of N % (4–2)	Balance
			1	2	3	4	5	6
Fraction I. Extracted with TCA	a	Free ammonia (+glutamine)	50	*0·63*	256	*2·99*	**+2·36**	**+6·65**
	b	Non-protein-N (not including 'a')	730	*9·23*	959	*11·19*	**+1·96**	
Fraction II. Extracted with organic solvents	c	'Lipid'–N	540	*6·83*	785	*9·16*	**+2·33**	
Fraction III. Extracted with 10% (w/v) NaCl	d	'Nucleic acid'–N	1595	*20·17*	1525	*17·80*	**−2·37**	**−6·65**
Fraction IV. Hydrolysed with 20% (w/v) HCl	e	'Protein'–N	4740	*59·95*	4800	*56·03*	**−3·92**	
Fraction V. HCl-insoluble	f	Humins	251	*3·17*	241	*2·81*	**−0·36**	
Total			7906	*100·00*	8566	*100·00*	**0**	**0**

RESULTS

(a) *Experiments* in vitro

In order to illustrate in detail the method used for calculating the results, a typical experiment dealing with the changes of nitrogen distribution brought about by incubating guinea pig brain cortex slices in the absence of added substrate is presented in Table 1.

It is shown that incubation of brain cortex slices is accompanied by an increase in ammonia, non-protein nitrogen (exclusive of ammonia) and 'lipid' nitrogen, associated with a decrease in 'protein' and 'nucleic acid' nitrogen. The shift of nitrogen within

the different fractions is quantitatively accounted for. The mean percentage fluctuation of the nitrogen distribution within the different fractions during incubation obtained from five separate trials is presented in Table 2.

In the NaCl-extract ('nucleic acid' fraction) the question arises whether the decrease of nitrogen in this fraction results from nucleic acid catabolism or degradation of other nitrogenous compounds present. The decreased optical density observed in the

TABLE 2.—NITROGEN CHANGES IN TCA-SOLUBLE, 'LIPID', 'NUCLEIC ACID' AND 'PROTEIN' FRACTIONS OF GUINEA PIG BRAIN CORTEX SLICES DURING AN INCUBATION OF 5 hr IN ABSENCE OF ADDED SUBSTRATE

Results are expressed as percentages of total nitrogen and the means of five experiments ±S.E.M. are given. Each experiment represents a batch of slices from six Warburg vessels, each vessel containing 60–100 mg wet weight of slices. The results of analysis of pentoses in rows 'g' and 'h' are expressed as percentages of initial values; means of four experiments.

		Initial	Final	Difference	Balance
a	Free ammonia (+glutamine)	0·55 ± 0·08	2·73 ± 0·03	**+2·18**	**+8·64**
b	Non-protein-N (not including 'a')	7·14 ± 0·54	11·14 ± 0·24	**+4·00**	
c	'Lipid'–N	5·27 ± 0·57	7·73 ± 0·45	**+2·46**	
d	'Nucleic acid'–N	21·77 ± 1·49	17·65 ± 0·31	**−4·12**	**−8·63**
e	'Protein'–N	61·87 ± 1·59	57·92 ± 0·62	**−3·95**	
f	Humins	3·39 ± 0·09	2·83 ± 0·17	**−0·56**	
g	TCA-soluble 'pentoses'	100·00 ± 3·2	62·10 ± 3·4	**−37·9**	$P^* < 0{\cdot}01$
h	NaCl-soluble 'pentoses'	100·00 ± 5·5	82·00 ± 4·3	**−18·0**	$P^* < 0{\cdot}01$

* Student's *t*-test of significance.

ultraviolet demonstrates the degradation of nucleic acids (Fig. 1). Degradation of other nitrogenous substances cannot be excluded.*

A significant decrease of orcinol-positive substances in the NaCl and TCA extract was observed. These results suggest that utilization of endogenous bound pentoses (orcinol-positive substances) is related to the endogenous metabolism of brain cortex slices (Table 2, g and h).

It was previously observed that an increase in K^+ concentration in the medium decreases the rate of ammonia formation in brain slices (VRBA *et al.*, 1957, 1958).

* This method of extraction of nucleic acids was selected because it permitted simultaneous determinations of pentoses, nitrogen and absorption in the ultraviolet. Hot $HClO_4$-extracts contain substances which interfered with the measurement of ultraviolet-absorption of nucleic acids. For similar reasons the extraction of nucleic acids with hot TCA was avoided.

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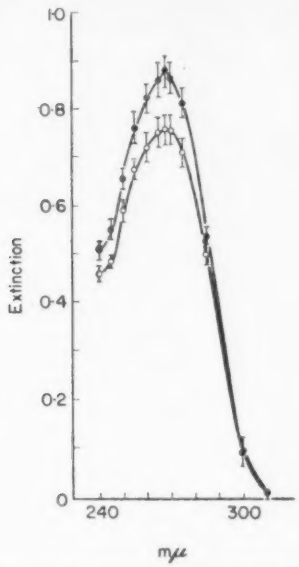

FIG. 1.—Ultraviolet absorption of the NaCl extract of guinea pig brain cortex slices. Each point is the mean of four experiments ± S.E.M: ●—● fresh slices; ○—○ after 5 hr incubation 37°.

TABLE 3.—EFFECT OF K^+ ON THE NITROGEN DISTRIBUTION IN GUINEA PIG BRAIN CORTEX SLICES DURING AN INCUBATION OF 5 HR IN ABSENCE OF ADDED SUBSTRATE

The figures are obtained by analysis of a combined batch of slices from six Warburg vessels; each vessel containing 60–80 mg fresh weight of slices. K^+ was increased by replacing NaCl by KCl.

	Fraction	Experiment 5										
		Initial		Final (K^+ = 6·25 mM)				Final (K^+ = 131·5 mM)				Effect of increased K^+ (9–5)
		μg N	N (% of total N)	μg N	N (% of total N)	Change (4–2)	Balance	μg N	N (% of total N)	Change (8–2)	Balance	
		1	2	3	4	5	6	7	8	9	10	11
a	Free ammonia	20	*0·31*	175	*2·25*	+1·94	+7·93	100	*1·48*	+1·17	+7·93	−0·77
b	Non-protein-N (not including 'a')	400	*6·28*	805	*10·34*	+4·06		740	*10·98*	+4·70		+0·64
c	'Lipid'-N	407	*6·39*	647	*8·32*	+1·93		570	*8·45*	+2·06		+0·13
d	Proteins + nucleic acids + humin	5540	*87·01*	6151	*79·08*	−7·93	−7·93	5331	*79·08*	−7·93	−7·93	+0·0
e	Total	6367	*100·00*	7778	*100·00*	0	0	6741	*100·00*	0	0	0

The effect of increased K^+ concentration on nitrogen metabolism in brain cortex slices (5 hr incubation) is shown in Table 3 in detail for one trial.

The results demonstrate that K^+ has an inhibitory effect on ammonia formation: no effect of K^+ on the catabolism of nitrogenous compounds of high molecular weight and 'lipid'-nitrogen accumulation was observed. Inhibition of ammonia

accumulation associated with increased K^+ concentration in the medium is related to an increase in non-protein nitrogen. Table 4 presents the mean values obtained from four other separate trials of 4 hr duration which agree with the latter observations. Table 4 also reveals that an increase in the K^+ does not affect the rate of utilization of TCA-soluble pentoses (orcinol-positive substances).

(b) *Experiments* in vivo

Previous work demonstrated a decrease of nitrogen in the 'protein fraction' of the rat brain during physical exercise (VRBA, 1955*b*; 1957). In these experiments the

TABLE 4.—EFFECT OF K^+ ON NITROGEN DISTRIBUTION AND UTILIZATION OF 'PENTOSES' DURING AN INCUBATION OF 4 hr IN ABSENCE OF ADDED SUBSTRATE

Results are expressed as percentages of total nitrogen and the means and standard errors are calculated (average of four experiments). Each experiment represents a combined batch of slices from three Warburg vessels, each vessel containing 100–120 mg fresh weight of slices. Thus each of four experiments is the result of analysis of 300–360 mg of slices (about six slices were obtained from various parts of the brain and from various individual guinea pigs).

Results in row e are expressed as percentages of the extinction of the initial values.

K^+ increased by replacing NaCl by KCl. t = Student's t-test.

	Fraction	1. Initial	2. Final, (K^+ = 6·25 mM)		3. Final, (K^+ = 131·5 mM)		4. Effect of K^+	
		TCA added in zero time (1)	4 hr's incubation (2)	Change (2–1)	4 hr's incubation (3)	Change (3–1)	Change (3–2)	t*
a	Free ammonia (+glutamine)	0·60 ± 0·02	2·36 ± 0·07	+1·76	1·48 ± 0·08	+0·88	**−0·88**	**8·27**
b	Non-protein-N (not including 'a')	7·37 ± 0·65	9·59 ± 0·40	+2·22	11·22 ± 0·38	+3·85	**+1·62**	**2·96**
c	'Lipid'–N	5·89 ± 0·24	7·42 ± 0·43	+1·53	6·78 ± 0·31	+0·89	−0·64	1·21
d	Nucleic acids + proteins + humine	86·14 ± 0·61	80·61 ± 0·36	−5·53	80·52 ± 0·57	−5·62	−0·08	0·13
e	TCA-soluble 'pentose'	100·00 ± 2·0	53·90 ± 1·8	−46·10	52·60 ± 0·4	−47·40	+1·3	0·71

* Significant effects of increased K^+ in the medium are printed in bold figures. Difference between columns 3 and 2 tested with Student's t-test.

'protein fraction' represented the brain tissue residue after extraction with TCA and organic solvents, thus including the nucleic acids. It is not clear from which part of this residue the nitrogen loss occurred. Since ammonia formation *in vitro* is connected with the degradation of nucleic acids and proteins, and since brain activity is associated with ammonia formation, we have undertaken the study of nitrogenous substances in the rat brain during physical exercise in further detail. The observation that endogenous pentoses (orcinol-positive substances) are utilized in brain slices *in vitro*, led to a study of bound pentoses *in vivo*. The changes in 'lipid' and 'protein' nitrogen in the brain during physical exercise were also of interest. GEIGER and co-workers showed that electrical stimulation of the cat brain is accompanied by an

increase in non-protein nitrogen and a decrease in lipid and nucleic acid nitrogen (GEIGER, DOBKIN, and MAGNES 1953; GEIGER, YAMASAKI and LYONS, 1956; for review see GEIGER, 1958).

The results of the present experiments are presented in Table 5 and Fig. 2 indicating that exertion is associated with an increase of free glutamine in the rat brain and a decrease of 'protein' and 'nucleic acid' nitrogen. This is in agreement with

TABLE 5.—CHANGES OF NITROGENOUS COMPONENTS AND PENTOSES IN RAT BRAIN DURING STRESS (PHYSICAL EXERTION BY SWIMMING)

Each figure in the table represents an average of four experiments ±S.E.M. Each analysis (1 experiment) was performed on a pooled sample of three brains. Thus each figure in the table represents the average of twelve brains.

Results in rows a–e expressed in μmoles N/g fresh weight of brain. Results in rows f–h expressed as percentages of controls. Average of control experiments (twelve brains) = 100.

	Fraction	Expt. A			Expt. B		
		Controls	Fatigued	Average difference (%)	Controls	Fatigued	Average difference (%)
a	Amide–N (TCA-soluble)	6·5 ± 0·7	7·3 ± 0·5	±12·3	5·2 ± 0·04	5·7 ± 0·07	±9·6
b	Non-protein–N (not including 'a')	87·7 ± 2·6	81·5 ± 2·2	−7·1	97·0 ± 1·9	97·8 ± 1·5	+0·8
c	'Lipid'–N	122·1 ± 5·7	105·4 ± 2·4	−13·7	140·6 ± 4·6	138·8 ± 2·3	−1·3
d	'Nucleic acid'–N	146·5 ± 2·8	133·8 ± 1·7	−8·6	147·0 ± 3·9	145·5 ± 3·8	−1·7
e	'Protein'–N	1134·0 ± 39·0	1114·0 ± 16·0	−1·8	996·0 ± 4·6	955·0 ± 7·8	−4·1
f	TCA-soluble pentoses	100·0 ± 2·7	95·8 ± 2·3	−4·2	100·0 ± 1·0	99·8 ± 0·14	−0·25
g	NaCl-soluble 'pentoses'.	100·0 ± 1·6	86·2 ± 0·5	−13·8	100·0 ± 1·0	95·2 ± 0·8	−4·8
h	Absorption of the NaCl-extract at 260 mμ	100·0 ± 3·3	90·9 ± 1·3	−9·1	100·0 ± 2·6	97·2 ± 1·3	−2·8

As only the trend of results was considered in the discussion, statistical evaluation was omitted.

previous observations (VRBA, 1954*b*; 1955*b*). Increased catabolism of nucleic acids is also indicated by decreased absorption of the NaCl-extract at 260 mμ, and a decreasing amount of orcinol-positive substances (pentoses) was observed in both groups of animals. The 'lipid nitrogen' and non-protein nitrogen (exclusive of glutamine) did not increase.

The *in vivo* results are not sharply defined. This is to be expected even if the technical difficulties are overcome, because the homeostatic mechanisms of the intact healthy animal will prevent marked deviations from the normal. In spite of these difficulties the results obtained in both groups of animals studied *in vivo* show that 'protein' nitrogen, 'nucleic acid' nitrogen and 'pentoses' follow a trend which corresponds with the statistically significant results obtained *in vitro*. This was also true in the group of rats picked at random (Expt. A) which on the whole had a larger standard deviation for values obtained than in the case of those animals which were bred and selected for homogeneity (Expt. B).

The brains of fatigued rats contain a reduced amount of orcinol-positive substances (endogenous bound pentoses), but it should be mentioned that in previous experiments a reduced glycogen, glucose and lactic acid content was demonstrated under similar

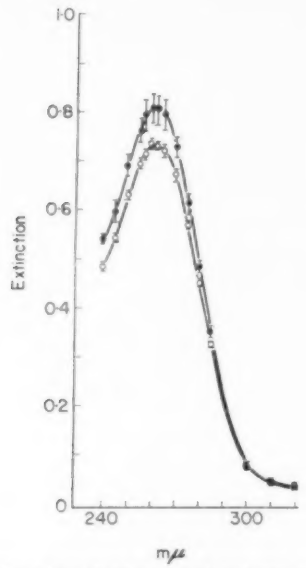

FIG. 2.—Ultraviolet absorption of the NaCl extract of rat brain. Each point is the mean of four experiments (twelve brains) ±S.E.M. (Series A): ●—● control; ○—○ after 4·5 hr swimming.

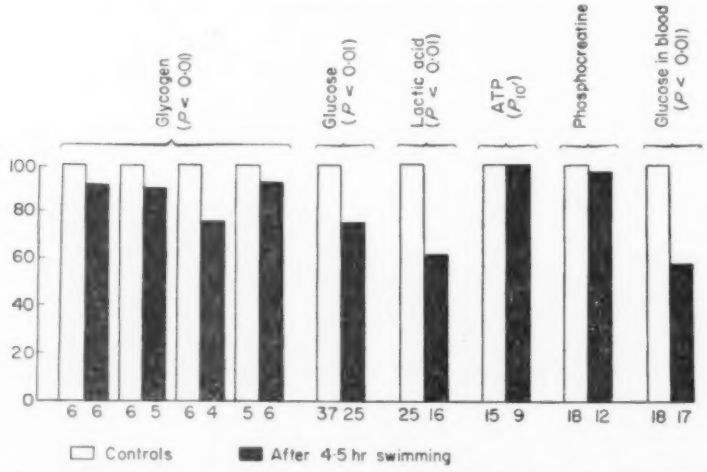

FIG. 3.—Changes in concentration of glycogen, glucose, lactic acid, ATP (P_{10}) and phosphocreatine in rat brain during physical exercise (4·5 hr swimming). Results are expressed as percentages of controls. The columns represent mean values: the figure under each column indicates the number of brains analysed. The data are from another communication (VRBA, 1954*a*). Glycogen was estimated according to SEIFTER, DAYTON, NOVIC and MUNTWYLER (1950), glucose according to NELSON (1944), lactic acid according to BARKER and SUMMERSON (1941), and phosphocreatine according to ALEXEJEVA (1951).

conditions (Fig. 3). Reduced concentrations of these three substances were also observed in brain cortex slices after respiration in the absence of added glucose by TAKAGAKI *et al.* (1957).

A comparison of Table 1 and Table 5 shows that brain fluctuations in 'lipid' nitrogen and non-protein nitrogen *in vivo* were opposite to those observed *in vitro*.

DISCUSSION

Carbohydrate and nitrogen metabolism in brain cortex slices

Our results indicate that the catabolism of endogenous bound pentoses and nitrogenous compounds of high molecular weight are related to ammonia formation but are independent of increased potassium ion concentration, which inhibits the formation of ammonia while increasing the rate of accumulation of NPN (Table 4). It has been previously shown that increased K^+ decreases the oxygen consumption of brain slices in the absence of added substrate (ELLIOT, 1955; VRBA and FOLBERGROVÁ, 1958*a*). The above observations suggest that during the catabolism of nitrogenous compounds of high-molecular weight an intermediary TCA-soluble nitrogenous substance (or substances) is formed, which may be further oxidized with the release of ammonia. This oxidation associated with the release of ammonia is inhibited by increased K^+ concentration in the medium. This hypothesis accounts for the fact that an increased concentration of K^+ in the medium inhibits ammonia formation and oxygen uptake by endogenous substrates while at the same time promoting the accumulation of non-protein nitrogen (excluding ammonia).

The oxygen uptake by brain cortex slices in the absence of added substrate may be due to the oxidation of endogenous carbohydrates including pentoses and nitrogenous substances.

The decrease of nucleic acid nitrogen during incubation coincides with the increase of 'lipid' nitrogen and liberation of ammonia. It is possible that 'lipid' nitrogen formation is a link in the chain of events leading to free ammonia formation.

A comparison of processes in vivo *and* in vitro

Ammonia formation in brain slices is studied on partially autolysed tissue and it can be related to processes *in vivo* only with extreme caution. Nevertheless a close connexion between ammonia formation in brain cortex slices and ammonia formation induced by the stimulation of intact components of the CNS has been suggested (WEIL–MALHERBE and GREEN, 1955; VRBA *et al.*, 1958). During the incubation of brain cortex slices an increased catabolism of nucleic acids and proteins occurs (Table 1, Fig. 1). Similar processes were observed in the brain of rats after physical exercise (Table 5, Fig. 2). Increased nucleic acid and protein catabolism in nervous tissue following stimulation has been demonstrated many times (HYDÉN, 1943; 1947; 1952; 1955: GEIGER *et al.*, 1956; GEIGER, 1958). During incubation of brain cortex slices (in absence of added substrate) utilization of endogenous glycogen, glucose and lactic acid account for a fraction of the oxygen uptake (TAKAGAKI *et al.*, 1957). It may be assumed that other endogenous substrates are oxidized and that a similar process probably occurs during stimulation of the isolated superior cervical ganglion, the perfused cat brain and other components of the nervous system, where it has been shown that the oxygen uptake is not accounted for by glucose utilization (cf. LARRABEE *et al.*, 1957; GEIGER *et al.*, 1952; ALLWEISS and MAGNES, 1958; GERARD, 1932; MULLINS, 1953).

The results presented in Tables 2 and 5 indicate that orcinol-positive substances (presumably endogenous bound pentoses) are utilized *in vivo* and *in vitro* and probably are responsible for a part of the oxygen consumption not otherwise accounted for.

In two instances the *in vivo* processes showed an opposite trend to those observed *in vitro*. Non-protein nitrogen (excluding ammonia) increased *in vitro*, but after

physical exertion, the non-protein nitrogen concentration in the brain tended to decrease. 'Lipid' nitrogen increased *in vitro*, but an opposite tendency was observed after physical exertion. A decrease of 'lipid' nitrogen in the intact brain after stimulation was also noted by other authors (ABOOD and GEIGER, 1955; GEIGER *et al.*, 1956). An increase in non-protein nitrogen in the intact brain following short electrical stimulation has been reported (GEIGER *et al.*, 1953).

The observed differences do not necessarily imply different mechanisms, because metabolic end-products *in vivo* may be rapidly removed and on the other hand substances catabolized may be speedily replaced. Technical objections to this conclusion apart from the involvement of a lack of homeostasis *in vitro* may be raised. Yet the writers concur with the view of WEIL–MALHERBE (1950), who states that: 'On the whole it seems improbable that the large ammonia formation of brain slices can be accounted for by low molecular cell constituents pre-formed *in vivo*. If it is derived from high molecular cell constituents, such as proteins, polynucleotides or lipids, the process might be described as autolysis. Yet autolysis only means the occurrence of exergonic reactions without their endergonic counterpart, and each of these exergonic reactions is probably of significance for vital processes'.

The observation that ammonia formation in brain cortex slices may be accounted for by catabolic processes in the nucleic acid and protein fraction does not exclude the participation of protein-bound amide in these processes. Various aspects of the metabolic activity of protein-bound amide have been discussed elsewhere (VRBA, 1955*a*, 1955*c*; VRBA *et al.*, 1957, 1958; WEIL–MALHERBE and DRYSDALE, 1957; KLEIN, 1957; GERSHENOVITSCH and KRITSHEVSKAYA, 1957; CLARKE, NEIDLE, SARKAR and WAELSCH, 1957). It should be emphasized that ammonia formation in brain slices is a complex process (cf. WEIL–MALHERBE and GREEN, 1955; VRBA *et al.*, 1958).

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SUMMARY

(1) The incubation of guinea pig brain cortex slices in absence of added substrate is associated with an increase of free ammonia, non-protein nitrogen and 'lipid' nitrogen. This increase is quantitatively accounted for by a decrease of nitrogen in the nucleic acid and protein fractions. Simultaneously, orcinol-positive substances diminish, indicating the utilization of endogenous bound pentoses.

(2) An increase of the K^+ concentration in the incubation medium decreases the rate of ammonia formation which is associated with an increase in the non-protein nitrogen fraction (excluding ammonia). The logical conclusion from this observation is that the intermediary non-protein nitrogen components are precursors of ammonia and that the oxidative release of ammonia is blocked by K^+ ions.

(3) The concentration of TCA-insoluble nitrogenous substances (nucleic acids and proteins) and orcinol-positive substances (presumably bound pentoses) in the rat brain tended to decrease during physical exercise. Simultaneously, an increase of TCA-soluble glutamine was observed.

(4) Our results suggest that the catabolism of proteins and nucleic acids, utilization of 'non-glucose substrates' and ammonia formation are interdependent processes *in vitro* and that the *in vivo* mechanisms may be of a similar nature.

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LOCALIZATION OF DOPAMINE-β-OXIDASE IN BRAIN

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THE sympathetic hormone norepinephrine has been shown to be derived from tyrosine and it is generally recognized that the immediate precursor is 3:4-dihydroxyphenylethylamine (dopamine). Conversion of the latter compound to norepinephrine in isolated peripheral tissues has been demonstrated many times. The presence of both dopamine and norepinephrine in the central nervous system suggested that this conversion also takes place in brain. The data presented in this report indicate that not only does brain convert dopamine to norepinephrine but that the enzyme responsible for this conversion, dopamine-β-oxidase, is localized in those areas of the brain which are richest in norepinephrine and other catalysts and intermediates involved in its synthesis.

MATERIALS AND METHODS

Preparation of [1-^{14}C]dopamine (0·62 mc/m-mole) was described previously (ROSENFELD, LEEPER and UDENFRIEND, 1958). It was purified by chromatography on paper by use of phenol—0·01 N-HCl (80 gm : 20 ml), saturated with SO_2, and kept as a solution in 0·01 N-HCl. The enzymic materials used in these studies were prepared from brain and various portions of brain and adrenal medulla by homogenization with an equal part of isotonic KCl.

Incubation mixtures consisted of 1 ml of homogenate (0·5 gm of tissue), 0·1 ml of [1-^{14}C]dopamine (200 μg; sp. act. 4110 c.p.m./μg), and 1·0 ml of 0·1 M-phosphate buffer, pH 7·4. Although the cofactor requirements of dopamine-β-oxidase are as yet unknown there have been indications of stimulation by pyridine nucleotide and adenosine triphosphate (NERI *et al.*, 1956). The incubation mixture was fortified with adenosine triphosphate, 1 μmole nicotinamide, 10 μmoles pyridoxal phosphate, 1 μmole di- and tri-phosphopyridine nucleotides, 1 μmole each; flavin mononucleotide, 0·5 μmoles; flavin adenine dinucleotide, 0·5 μmoles; cytochrome *c*, 100 μg; coenzyme A, 0·5 μmoles; citrate, 1 mg; glucose, 2 mg; $MgSO_4$, 50 μmoles; and it contained 1 mg of carrier norepinephrine. The final volume was 3·5 ml. Incubations were carried out on a Dubnoff metabolic incubator in air at 37° for 3 hr. They were stopped by the addition of 1 ml of 40% w/v trichloroacetic acid and the norepinephrine was isolated as described previously (UDENFRIEND and WYNGAARDEN, 1956). This involved two chromatographic separations with the phenol solvent system (above) followed by fluorometric assay to determine the recovery of carrier norepinephrine. An additional amount (20 mg) of non-radioactive norepinephrine was added to the norepinephrine isolated from the chromatograms as carrier, and the mixture was recrystallized to constant specific activity. In practice, five recrystallizations were found to be more than adequate. Samples were plated out on planchettes and radioactivity was measured in a Robinson gas-flow counter having a background of 2–3 c.p.m. (ROBINSON, 1950). The measured radioactivity was then corrected for self-absorption.

Approximately 15–25 per cent of the original norepinephrine was isolated in the overall procedure and the measured counts were corrected for the loss of carrier incurred during purification.

RESULTS AND DISCUSSION

Although the conversion of dopamine to norepinephrine is apparently enzymically catalysed, attempts to assay enzyme activity by standard procedures were not

successful owing to the low activity of the tissue catalysts and to technical problems of assay. For this reason radioactive methods were used.

In a typical experiment with dog hypothalamus, the total radioactivity in the enzymically formed norepinephrine was 4900 c.p.m. as compared to 432 c.p.m. for the boiled control. Although the radioactivity in the boiled control was relatively small, it was real since the reagents themselves yielded no radioactivity when carried through the experimental procedure. However, as has been reported elsewhere (SENOH, WITKOP, CREVELING and UDENFRIEND, in press) the material appearing in the boiled controls apparently represents 3:4:6-trihydroxyphenylethylamine which

TABLE 1.—DOPAMINE-β-OXIDASE CONTENT OF VARIOUS PORTIONS OF BRAIN AND ADRENAL MEDULLA

Species	Tissue	Norepinephrine formed μg/g/3 hr	
		Homogenate	Boiled control
Rat	Whole brain	0·26	0·04
Dog	Hypothalamus	2·18	0·24
	Caudate nucleus	1·60	0·18
	Cerebellum	0·08	—
	Cortex	0·004	0·008
	Adrenal medulla	2·34	0·20
Beef	Hypothalamus	1·96	0·24
	Caudate nucleus	2·14	0·08
	Cerebellum	0·08	—
	Adrenal medulla	1·28	0·08
Sheep	Adrenal medulla	0·68	0·02

can be formed non-enzymically from dopamine. The former compound is isomeric with norepinephrine and is not separated during the purification procedures utilized in the present study. Nevertheless, the additional material formed enzymically does represent norepinephrine.

The data presented in Table 1 indicate that brain homogenates do contain appreciable amounts of dopamine-β-oxidase. In all instances, the enzymic activity was many times greater than the non-enzymic activity of the boiled controls. It is of interest that the most active areas of brain were comparable in activity to adrenal medulla even though adrenal medulla contains more than one-thousand times as much catecholamines as does brain. This may be taken as additional evidence that the large amounts of these amines in the adrenal gland are not indicative of their rate of synthesis *in vivo* (UDENFRIEND and WYNGAARDEN, 1956).

Of further interest is the finding that brain dopamine-β-oxidase is highly localized, large amounts being present in hypothalamus and caudate nucleus, little in cerebellum, and no detectable amounts in cortex. The latter two tissues are known to contain little catecholamine whereas the concentration in hypothalamus is relatively high

(VOGT, 1954; BOGDANSKI, BRODIE, KUNTZMAN and SHORE, submitted for publication). Although caudate nucleus contains little norepinephrine it is rich in dopamine (CARLSSON, in press). The finding of both dopamine and dopamine-β-oxidase in this tissue indicates that it is certainly capable of synthesizing norepinephrine.

The dopamine-β-oxidase content of the brain stem areas and caudate nucleus is much lower than the dopa decarboxylase activity, about <1 μg/g/hr as compared with >100 μg/g/hr (BOGDANSKI, BRODIE, KUNTZMAN and SHORE, submitted for publication). More detailed measurements of dopamine-β-oxidase activity in the central nervous system along with the other catalysts and intermediates should make it possible to map out in detail those areas of the brain in which norepinephrine plays its physiologic role. Such information may eventually prove useful in determining the exact function of the catechol amines in the central nervous system and to determine which biosynthetic steps may be rate limiting with respect to norepinephrine formation and release.

SUMMARY

Enzymic conversion of 3:4-dihydroxyphenylethylamine to norepinephrine (dopamine-β-oxidase) has been shown to be catalysed by homogenates of brain. Dopamine-β-oxidase is found in high concentration in hypothalamus and caudate nucleus, little activity being present in cortex and cerebellum.

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METABOLISM OF MYELIN LIPIDS: ESTIMATION AND SEPARATION OF BRAIN LIPIDS IN THE DEVELOPING RABBIT

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RADIOACTIVE cholesterol has been shown by DAVISON, DOBBING, MORGAN and PAYLING WRIGHT (1958, 1959*a*) to be incorporated in the central nervous system when it is administered to immature animals and to remain in that organ for more than a year. These investigations have now been extended to a similar study (DAVISON, MORGAN, WAJDA and PAYLING WRIGHT, 1959*b*) in which [3-^{14}C]serine has been used as the metabolic precursor of sphingolipids and other lipid components of the myelin sheath. The present paper describes and evaluates the methods needed in that study for the quantitative separation of the individual lipids from the crude brain extract. It further records some observations on their differing rates of deposition in the central nervous system of rabbits during growth.

MATERIALS AND METHODS

The Copenhagen White rabbits used in this work were killed by intravenous injection of pentobarbitone. The brain was removed and homogenized with 20 times its volume of chloroform–methanol (2 : 1 v/v). After filtration, the moist extract was evaporated to dryness three times under reduced pressure to break down proteolipids A and B (FOLCH and LEES, 1951). The lipids were then extracted with chloroform–methanol (2 : 1 v/v), filtered free from protein and the extract adjusted to the original volume. This protein-free extract was given three washes, first with water and then twice with the 'upper phase' solution described by FOLCH, LEES and SLOANE STANLEY (1957). Such treatment removes water-soluble material, particularly amino acids, salts and ganglioside. The lower phase was then used for the following analyses and separations.

Chromatographic separation. Lipids were separated on an alumina column (RHODES and LEA, 1957) by the technique of LONG (personal communication). In this method, not more than 500 mg of lipid in 5 ml chloroform–methanol (98 : 2 v/v) is placed on a column of 50 g alumina (British Drug Houses, Poole). The column is successively eluted with 55 ml of chloroform–methanol (98 : 2 v/v); 600 ml of chloroform–methanol (1 : 1 v/v); 700 ml of chloroform–methanol–water (7 : 7 : 1 v/v) and finally with 500–1000 ml of chloroform–ethanol–water (2 : 5 : 2 v/v).

Dry weight. Samples (approximately 100 mg) were heated at 110° for 2 hr.

Total lipid. Suitable volumes of the protein free chloroform–methanol extract were evaporated to dryness. Total lipid determined in this way also contains some water soluble impurities.

Total phospholipid. Lipid samples were oxidized with 1 ml perchloric acid until clear and phosphorus determined as described by STRICKLAND, THOMPSON and WEBSTER (1956).

Cholesterol and its esters. The method described previously by DAVISON *et al.* (1958) was used.

Nitrogen. Extracts were digested in sulphuric acid with a catalyst. After making them alkaline, ammonia was distilled off and estimated with Nessler's reagent (BALLENTINE, 1957).

Primary amino groups. The method of MOORE and STEIN (1954) was used. Cerebroside samples were hydrolysed for 90 min with 6 N-H_2SO_4 at 100°.

Sphingomyelin (phosphosphingosides). Lipid samples were hydrolysed with N-KOH for 24 hr at 37°. After neutralization the unhydrolysed lipids were treated for 2 hr with 0·5 ml of 50% (w/v) trichloroacetic acid to decompose plasmalogens and precipitate the sphingomyelin. After filtering,

total phosphorus was determined on the filtrate. Unhydrolysed lipid phosphorus multiplied by a factor of 25 gave the sphingomyelin content (SCHMIDT, BENOTTI, HERSHMAN and THANNHAUSER, 1946; BRANTE, 1949). For analysis of whole lipid extracts this value of sphingomyelin includes those phospholipids unhydrolysed by alkali and insoluble in trichloroacetic acid (see BRANTE, 1949; DAWSON, 1954; SCHMIDT and SPENCER, 1957). However after separation of the lipid extract on the column the cephalins are eluted in the final fraction, and true sphingomyelin is given by the alkali-stable phospholipid present in the lecithin–sphingomyelin fraction. Not only is ninhydrin reacting material absent from this fraction but paper chromatograms indicate that only two components are present.

Lecithin and cephalin. Total choline was determined by the method of SMITS (1957) on extracts previously hydrolysed with saturated barium hydroxide for 90 min at 100° (BRANTE, 1949). Lecithin was represented by the choline (multiplied by 6·4) liberated by hydrolysis with 1 N-KOH for 24 hr at 37°. Total phospholipid less the sum of sphingomyelin plus lecithin equals the sum of the cephalins.

Plasmalogens. The method of LEUPOLD and BUTTNER (1953) with a slight modification suggested by Dr. SLOANE STANLEY was used. Tetradecyl aldehyde (Theodore St. Just & Co. Ltd., Whitefield, Manchester) used as a standard, was kindly supplied by Dr. SLOANE STANLEY. The calculation of plasmalogen content is based on a molecular weight of 685.

Cerebroside. The following method is based on those of EDGAR (1956) and of SVENNERHOLM (1956). Suitable volumes of lipid extracts, free from gangliosides, were evaporated to dryness. The residue was hydrolysed in 3 ml 6N-H_2SO_4 at 100° for 70 min. The hydrolysate was adjusted to 6 ml with water and then extracted with 5 ml of chloroform. Two ml of the aqueous layer was then transferred to a pyrex boiling tube, cooled in ice. Next, 1 ml of orcinol reagent (2 g orcinol dissolved in 100 ml of 20% H_2SO_4) was added, followed by 4 ml concentrated H_2SO_4. The mixture was stirred in the cold and the tubes then heated at 100° for 25 min. After cooling to room temperature, the optical density of the solution was determined at 505 mμ. The galactose concentration was converted to cerebroside content by multiplying by 4·55.

RESULTS

Separation of lipid fractions by column chromatography. From the concentrated extracts prepared from the brains of a number of rabbits, aged from 1 day to 2 years, individual lipids were separated on a column and analysed by the methods described above. The different solvent mixtures employed eluted in succession the following lipids: cholesterol; sphingomyelin and lecithin; cerebroside and cephalins. The mean percentage recovery calculated on the basis of determinations on the original extract for each group of lipid was found to be 84 per cent for cholesterol, 80 per cent for sphingomyelin, 86 per cent for lecithin, 84 per cent for cerebroside and 73 per cent for cephalin. Choline and phosphorus were absent in the cholesterol fractions and only traces of phosphorus were present in the separated cerebroside. It should be noted that in these separations the alumina used was supplied by British Drug Houses Ltd.

To investigate further the adequacy of this fractionation method for the separation of individual lipids, two experiments were carried out. The first was on ^{32}P-labelled phospholipids and the second on cerebroside. In the former, a chloroform–methanol extract from a rat brain, injected intraperitoneally 5 days before with 5 μC of ^{32}P as phosphate, was diluted with 400 mg of brain lipid similarly extracted from a 28 day old rabbit. The lipids were fractionated by the above method and their radioactivities determined in a liquid-type M6 (20th Century Electronics Ltd.) β-counter. The results are set out in Table 1, and show that the second and final fractions from the column contained all the recovered phospholipid; the cholesterol and cerebroside fractions contained virtually none of the isotope.

In the second experiment, the lipid extracts from the brains of six rabbits of

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TABLE 1.—RADIOACTIVITY OF LIPID FRACTIONS SEPARATED ON AN ALUMINA COLUMN

Total radioactivity		*Fraction* 1	2	3	4	Recovery
		Cholesterol	Lecithin + Sphingomyelin	Cerebroside	Cephalin	
(counts/min per specimen)	(41,900)	16	19,200	835	13,600	33,650
Percentage	(100%)	0%	46%	2%	32·5%	80·5%

various ages were individually separated on the alumina column. The cerebroside-containing fraction from each was then analysed for total dry weight, galactose, nitrogen, primary amine after hydrolysis, choline, neuraminic acid and phosphorus. These analyses, which are set out in Table 2, indicate that cerebroside separated in this way is 90–95 per cent pure, and that only small amounts of a non-choline-containing phospholipid (about 7 per cent) are present.

Changes in the lipid composition of the brain during development. The changes in the wet and dry weights of the brains of rabbits, together with total lipid contents,

TABLE 2.—ANALYSES OF CEREBROSIDE FRACTIONS

Six samples of cerebroside separated on an alumina column have been analysed by methods described in the text.

Age of rabbit (days)	Cerebroside (mg) calculated from: Dry wt of sample	Galactolipid	Nitrogen	Equivalents of primary amine (mg)
81	36·3	32·2	26·1	1·52
120	40·8	37·4	35·6	1·88
161	32·4	27·4	24·4	1·28
203	42·9	38	39·8	2·16
253	34	30	28·5	1·48
2 years	20	19·9	19·1	1·22

Choline and neuraminic acid were absent from all samples. Traces of phosphorus were present (mean values were about 7% phospholipid in each fraction). Primary amine equivalents, obtained after acid hydrolysis, are calculated in terms of leucine.

that take place during development are shown in Fig. 1 and Table 3. The lipids were extracted as described above and the washed protein-free extracts were analysed before separation on the column. Using these methods it is possible to obtain direct determinations of cholesterol, plasmalogen and cerebroside, and values for lecithin, cephalin and 'sphingomyelin' can be obtained by calculation. The changing values for each of these lipids (expressed as the total for the lipid in each brain) which occur during development are shown in Fig. 2 and Table 3. In some cases, these values

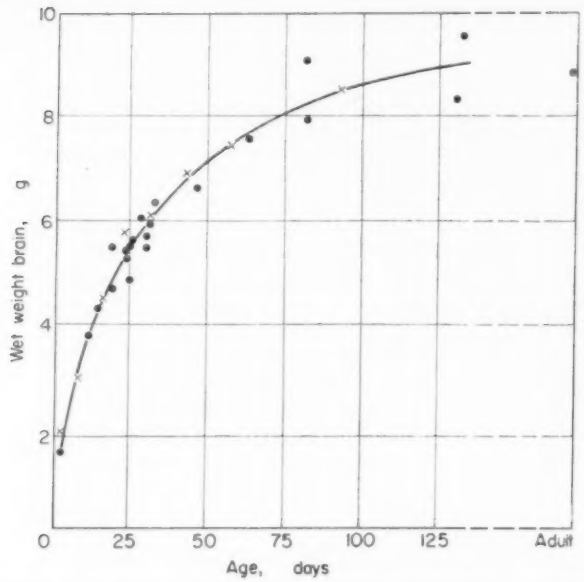

FIG. 1.—Changes in wet weight of rabbit brain during development. The present results (●) are compared with those of EDGAR (1956) (×).

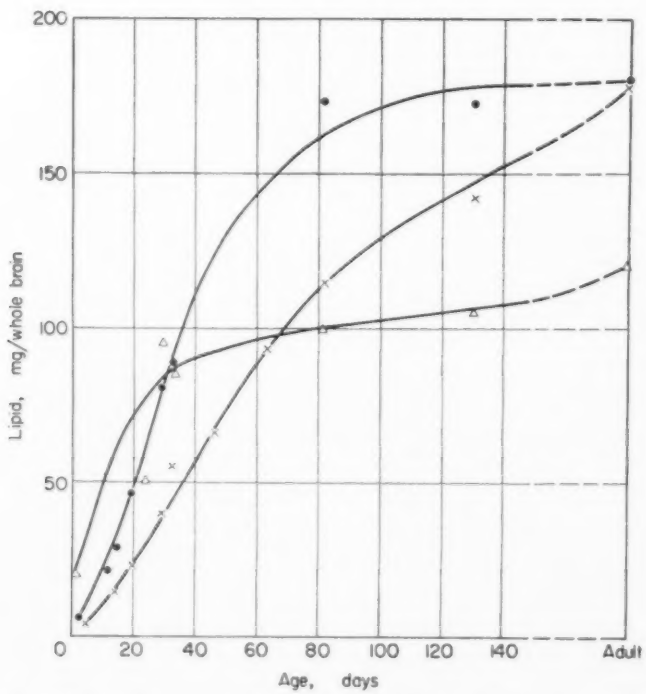

FIG. 2.—Changes in lipid content of the rabbits brain during development. Results for cholesterol (●), cerebroside (×) and lecithin (△) are shown.

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TABLE.—CHANGES IN LIPID CONTENT OF RABBIT BRAIN DURING DEVELOPMENT
The analytical methods used are described in the text. The values given are total lipid in each brain.

Age of rabbit (days)	Wet weight of brain (g)	Percentage dry weight (%)	Total lipid (mg)	Total phospholipid (mg)	Alkali labile phospholipid (mg)	'Sphingomyelin' (mg)	Plasmalogen (mg)
2	1·56	12	54	36	30	6	3
14	4·36	—	—	128	—	—	22
24	4·85	14	—	148	168	—	—
28	6·04	—	—	240	220	20	47
29	6·00	19	412	254	220	34	—
30	5·51	18	—	246	155	91	—
32	6·35	19	470	290	186	104	—
46	6·64	—	—	337	264	73	—
63	7·60	—	—	348	287	61	—
81	9·14	23	922	532	450	82	117
120	8·35	24	890	430	308	128	93
2 years*	8·92	22	987	488	383	105	119

* Mean of four results.

comprise more than one distinctive lipid: thus cephalin consists of the non-choline phospholipids such as phosphatidyl serine, ethanolamine and inositol, and plasmalogens.

DISCUSSION

Before beginning our investigation into the incorporation and fate of [3-^{14}C]serine in the central nervous system of developing rabbits (DAVISON *et al.*, 1959*b*), it was necessary to devise and test methods for the separation and estimation of the chief myelin lipids. The chromatographic techniques developed by LONG have been found very effective for this work. The recovery of cerebroside, cholesterol, sphingomyelin and lecithin was from 80 to 85 per cent of that present in the crude extract placed on the column, while rather lower recoveries of cephalin were obtained. These estimates obtained by chemical analyses were supported when a lipid extract containing ^{32}P phospholipid was separated by the same method: an overall recovery of 80 per cent of the initial radioactivity was obtained in two of the four fractions (lecithin, sphingomyelin and cephalins), while only traces of the isotope were detected in the cholesterol and cerebroside containing fractions.

The observations on the changing composition of the lipids during the period of myelination that have been recorded here confirm those of earlier workers (BRANTE, 1949; FOLCH, 1955; EDGAR, 1957; CUMINGS, GOODWIN, WOODWARD and CURZON, 1958). Of these investigators, only EDGAR employed the rabbit for his study, and unfortunately he omitted the estimation of cholesterol and plasmalogen in his otherwise comprehensive work. Furthermore, he did not distinguish between cerebroside and ganglioside. But within this more limited range, our analyses for total galactolipid, sphingomyelin, cephalin and lecithin agree well with his findings.

Although myelinated fibres have been detected in the medulla oblongata of the day old rabbit, myelination in other parts of the brain begins between 5 and 7 days after birth (EDGAR, 1957; DE ALMEIDA and PEARSE, 1958). As judged qualitatively by conventional histological methods, the process continues uninterruptedly thereafter to become complete some months later. In the rabbit, no such hiatus in development was observed either by EDGAR or ourselves comparable with that in the mouse

described by UZMAN and RUMLEY (1958), who recorded a temporary decrease in the weight of the brain in this species during the period of myelination some 16–23 days after birth. In the rabbit, there is no such break, for the brain grows steadily in wet and dry weight and lipid content up to about 80–100 days after birth. If in the rabbit brain as in that of the mouse, myelination is shortly preceded by a loss of water, the phenomenon might well be masked in records of increments of overall weight, since myelination in this larger species takes place more gradually and successively in different parts of the brain.

It is apparent from Fig. 2 and Table 3 that sphingomyelin and cerebroside values increase steadily from zero at birth apparently asymptotically until some 80–100 days afterwards. This is coincident with the period of myelination, and it is for this reason that these lipids are believed to be typical myelin lipids. It is also evident from Fig. 2 that cholesterol accumulates at a rate intermediate between that of the typical myelin lipid cerebroside and lecithin—a non-myelin lipid: a similar observation has been made in the mouse by FOLCH (1955). Although cholesterol is present in myelin it is not limited to this neural element; the dual role of cholesterol in the central nervous system is illustrated by our earlier recognition of two components of cholesterol metabolism with very different turnover rates—one slow and the other at a rate typical of other organs in the developing rabbit and chicken brains (DAVISON *et al.*, 1958, 1959*a*).

The studies recorded in this paper support the view of other previous workers, based on comparisons of myelinogenesis and chemical analyses, that cerebroside is the most characteristic and distinctive of the myelin lipids. Since the separation and quantitative recovery of cerebroside from the alumina column appeared to be satisfactory, its purity has been critically investigated. Chemical analysis has shown that it is about 95 per cent pure, any contamination being due to a non-choline-containing phospholipid. The application of these methods to the estimation of the turnover rates of the brain lipids in growing rabbits—injected when immature with [3-^{14}C] serine—is described in a separate paper (DAVISON *et al.* 1959*b*).

SUMMARY

1. The chromatographic method of LONG for the separation of lipids on an alumina column has been examined. Using this method, lipids from the brain of developing rabbits have been separated into cholesterol, cerebroside, choline phosphatide and non-choline phosphatide fractions.

2. Changes in the lipid content of the developing rabbit brain during myelination are described.

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METABOLISM OF MYELIN LIPIDS: INCORPORATION OF [3-^{14}C]SERINE IN BRAIN LIPIDS OF THE DEVELOPING RABBIT AND THEIR PERSISTENCE IN THE CENTRAL NERVOUS SYSTEM

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PREVIOUS studies with ^{14}C-labelled cholesterol (DAVISON, DOBBING, MORGAN and PAYLING WRIGHT, 1958, 1959) have shown that when it is injected into developing animals during the period of active myelination, this substance remains relatively inert for at least a year in the central nervous system, and, therefore, may well be retained indefinitely as a structural constituent of the myelin sheath. The practicability of likewise labelling other myelin lipids at their time of deposition in immature animals makes it possible to determine whether they, like cholesterol, persist in the brain and spinal cord long into the adult life of the animal. Were this so, it would provide further evidence that the myelin sheath, once formed in early life, should be regarded as among the more permanent tissue elements.

The sphingosine-containing lipids, cerebrosides and sphingomyelin, are among the most characteristic constituents of the myelin sheath (FOLCH, 1955; DAVISON and WAJDA, 1959). Because serine has been shown to be an effective precursor of sphingosine (SPRINSON and COULON, 1954; BRADY and KOVAL, 1958), we injected [3-^{14}C]serine into developing rabbits at a time when myelination is proceeding rapidly, and have followed for many months its persistence in the lipid fractions of the brain.

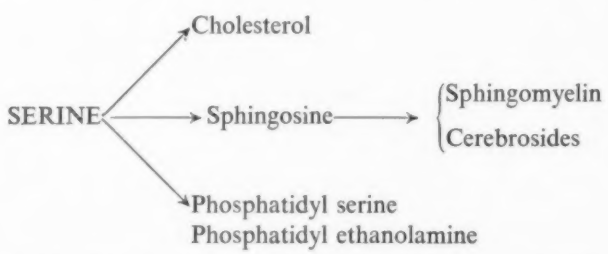

FIG. 1.—Possible paths for the anabolism of myelin lipids from serine.

The method of chromatographic separation of lipids as described by LONG (personal communication) has proved to be a useful technique for the fractionation of brain lipids, and especially for obtaining pure cerebrosides (see DAVISON and WAJDA, 1959). Employing this method, the brain lipids from the [3-^{14}C]serine injected rabbits have been separated, and the radioactivities of the cerebrosides, cholesterol, sphingomyelin, lecithin and cephalin determined at intervals of from one to 250 days

after the injection. In addition, an attempt has been made to ensure that the ^{14}C-label was in fact located in each of the lipid fractions isolated.

METHODS

Copenhagen White rabbits, 11 days old, from two litters, were given 16 μC of DL-[3-^{14}C]serine by intraperitoneal injection. At intervals, the animals were killed by intravenous injection of pentobarbitone. The brains were removed and homogenized in 20 times their volume of chloroform–methanol (2:1 v/v). Subsequent washing and removal of protein were carried out as previously described, and the same analytical and chromatographic methods were employed (DAVISON and WAJDA, 1959).

Radioactivity was determined in an Ekco type N612 liquid scintillation counter operated at room temperature. Using single-channel discrimination, the background was reduced to 25 counts/min with an overall efficiency of 35 per cent. The solvents used were toluene AR for the cholesterol fraction, while xylene (reagent grade, May and Baker) proved most suitable for all the other lipid fractions. The scintillator solute used was 2:5-diphenyloxazole (0·3% w/v). Suitable aliquots of brain lipid extracts were taken to dryness, and the residue dissolved in 20 ml xylene plus scintillator. Any quenching effect was corrected by the use of glyceryl tristearate-1-^{14}C as an internal standard. The reference standard was a ^{14}C-labelled methyl methacrylate preparation dissolved in toluene.

RESULTS

Radioactivity persisting in the total lipids of the brain. The radioactivity of the total lipids at intervals after a single injection of [3-^{14}C]serine into 11-day old rabbits is shown in Fig. 2. Crude lipid extracts, freed from protein, were then treated with

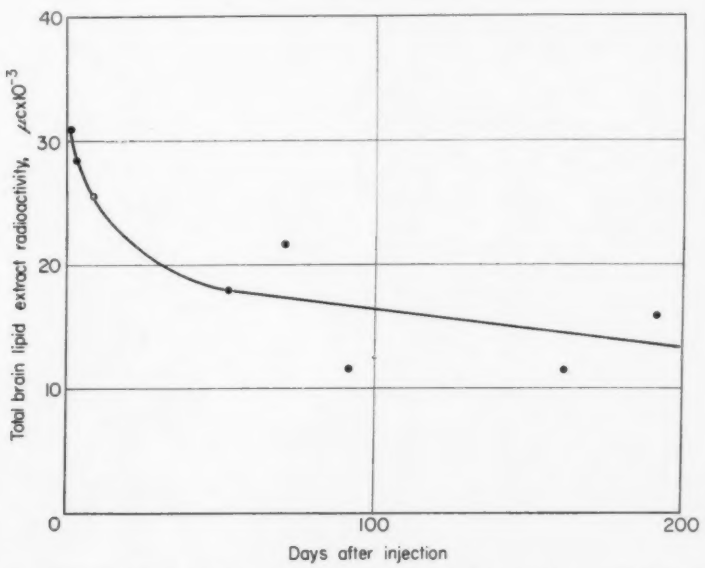

FIG. 2.—Radioactivity in total brain lipids at intervals after a single injection of [3-^{14}C]serine into 11 day old rabbits.

water as described by FOLCH, LEES and SLOANE STANLEY (1957). In the upper phase samples obtained after 8 days, little radioactivity was detectable. The lower phase, in which almost the whole of the radioactivity was present, was evaporated to dryness and its lipids separated by chromatography as previously described. The respective

radioactivities of the cholesterol, lecithin, sphingomyelin and cerebroside fractions are given in Table 1. About 85 per cent of the radioactivity of the total lipid applied to the column is recovered. Results for the cephalin fraction are not recorded because a progressive yellow discoloration in these samples produced variable results owing to quenching. However, in experiments in which measurements were made rapidly before the yellow colour had developed, the radioactivity of this fraction was comparable to those of the other fractions (see Table 2).

Radioactivity in the cholesterol and cerebrosides fraction. Portions of brain lipid extracts from the first (cholesterol) and third (cerebrosides) separations from the

TABLE 1.—RADIOACTIVITY IN THE LIPID FRACTIONS OF WHOLE RABBIT BRAIN AT INTERVALS AFTER THE INJECTION OF [3-^{14}C]SERINE

Days after injection	Brain wet weight (g)	Radioactivity (μc $\times$ 10^{-3})			
		Cholesterol	Cerebroside	Lecithin + Sphingomyelin	Sphingomyelin
1	3·81	6·33	1·89	10·31	—
3	4·36	4·62	4·28	10·61	3·96
8	4·75	4·19	3·06	7·52	3·68
52	7·60	3·98	3·82	4·17	4·12
70	8·50	4·62	7·08	4·90	—
93	9·00	5·49	4·03	4·42	2·17
109	8·54	6·53	7·79	5·73	4·27
161	7·92	3·16	3·89	0·91	0·94
192	9·52	5·84	6·35	3·20	1·32

TABLE 2.—RADIOACTIVITY IN CENTRAL NERVOUS SYSTEM AND SCIATIC NERVE OF A RABBIT 250 DAYS AFTER INJECTION WITH [3-^{14}C] SERINE

Sample	Weight (g)	Radioactivity (μc $\times$ 10^{-3})						
		Protein free extract	Washed lipid extract	Cholesterol	Cerebroside	Cephalin	Sphingomyelin	Lecithin
Brain white matter	4·61	6·32	6·00	1·15	2·64	0·66	0·67	0
Brain grey matter	4·36	1·41	1·38	0·61	0·34	0·14	0·17	0
Spinal cord	5·13	10·40	10·50	2·90	2·73	0·70	1·04	0·61
Sciatic nerve	0·61	0·65	—	—	—	—	—	—

column were evaporated to dryness and redissolved in xylene. Scintillator was then added. Xylene proved to be a suitable solvent as its counting efficiency was high. From Table 1, it can be seen that little change had occurred in the radioactivity in these two fractions—cholesterol and cerebrosides—nearly 200 days after the injection of the [3-^{14}C]serine.

In order to show that the radioactivity was due to incorporation of ^{14}C into sphingosine, the cerebrosides were hydrolysed by the method of BRADY and KOVAL (1958). The recovered sphingosine was purified. In two experiments, 91 and 95 per cent respectively of the original cerebroside radioactivity was found in the sphingosine moiety. Similarly, digitonin precipitation of the cholesterol removed the radioactivity from the first fraction.

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Radioactivity in the lecithin plus sphingomyelin fraction. The results set out in Table 1 show that there is a steady fall in the radioactivity for the first 50 days in the lecithin–sphingomyelin fraction. In order to discover whether this decline is attributable to loss of one or other of these lipids, this fraction was further fractionated by an adaptation of the alkaline hydrolysis method of SCHMIDT, BENOTTI, HERSHMANN and THANNHAUSER (1946). The non-hydrolysable sphingomyelin was precipitated with cold 10% (w/v) trichloroacetic acid, and the precipitate dried and dissolved in xylene for scintillation counting. From the results obtained, it appears that the radioactivity persists mainly in the sphingomyelin.

The distribution of radioactivity in brain, spinal cord and peripheral nerve. The distribution of radioactivity in the lipid fractions of the central nervous system and sciatic nerve trunk of a rabbit 250 days after its injection with [3-^{14}C]serine at 11 days old is shown in Table 2. The brain was dissected into 'white' and 'grey' matter (see DAVISON *et al.*, 1959) and these were treated separately. Substantially higher radioactivity was found in the 'white' than the corresponding 'grey' fractions of the brain. Furthermore, the radioactivity of the spinal cord and the sciatic nerve was comparable to that of the brain.

DISCUSSION

When [3-^{14}C]serine is injected into eleven-day old rabbits, it becomes quickly incorporated into the lipids of the brain and the resulting radioactivity persists there with little change for 250 days afterwards. If the crude lipid extracts from these brains are fractionated, the radioactivity is found to be associated with cholesterol, lecithin, sphingomyelin, cerebrosides and cephalin—evidence of the diverse metabolic pathways opened for the labelled serine. All these lipids, with the exception of lecithin, are believed to be important constituents of the myelin sheath, and it is particularly for this reason that we have undertaken a study of their persistence in the brain. Further, little is yet known, especially from long-term experiments, of the possible 'turnover rates' of these lipids in the central nervous system—a metabolic consideration of great significance as an indicator of the permanence or otherwise of the myelin sheath formed during development.

The present series of experiments extend and support our earlier studies on the fate of radioactive cholesterol after its administration parenterally into very young chickens and rabbits. One noteworthy feature which distinguishes the present serine experiments from those made earlier with cholesterol, however, is that in the former the radioactive cholesterol once synthesized persists with little or no loss, while in the latter, the amount of [4-^{14}C]cholesterol rose quickly to a peak and then declined slowly to reach a stable level some months later. Findings comparable to those with serine have been described by MCMILLAN, DOUGLAS and MORTENSEN (1957) with young rats of 200–250 g which had been injected with ^{14}C-labelled acetate: in their animals, too, there was no diminution in the amount of radioactivity in the cholesterol recovered from the brain 70 days after the injection. It would seem that radioactive cholesterol synthesized from some precursors exhibits a degree of metabolic inactivity that is characteristic of [4-^{14}C]cholesterol metabolism in the 'white' matter of the central nervous system (DAVISON *et al.*, 1959).

The radioactivity of the cerebrosides—perhaps the most distinctive of the myelin lipids—in the serine injected rabbits was found to persist in the central nervous system

for many months with little or no loss. This observation that the portion of the cerebroside molecule that has become labelled with ^{14}C is metabolically stable is indirectly supported by some findings by other workers. Thus, BURTON, SODD and BRADY (1958) inferred that the cerebrosides of the brain in adult rats undergo little metabolism from their finding that there was very little incorporation of labelled hexose into the neutral glycolipids of the organ.

Certain observations by other investigators, however, seem less immediately reconcilable with our finding that labelled cerebrosides persist for long periods in the central nervous system. RADIN, MARTIN and BROWN (1957) supposed on the basis of short term experiments (10 days) in which [^{14}C]galactose and [^{35}S]sulphate were used, that while cerebron sulphate underwent no metabolic breakdown in the brain of the young rat, cerebrosides did show some 'turnover'; their estimate for the half-replacement time was about 13 days. From some experiments on 14-day old rats given radioactive glucose and killed subsequently at intervals of up to 24 hours, BURTON and his associates suggested that neutral glycolipids undergo some metabolic breakdown in the brain. It seems likely, however, that by restricting themselves to experiments of such limited duration, both these groups of workers might well have failed to recognize the long-term persistence of this lipid in the central nervous system.

It has also been found in our experiments in which the animals have been immature at the time of the [3-^{14}C]serine injection that sphingomyelin—which with phosphatidyl serine has been identified as a myelin lipid (ROBINS, EYDT and SMITH, 1956)—and some of the 'cephalins', though not lecithin, undergo little 'turnover' in the brain.

Additional evidence that the lipids of the myelin sheath are relatively inert metabolically comes from the comparative distribution of radioactivity in the 'grey' and 'white' matters of the brains of the serine injected rabbits. It has been shown in an earlier paper (DAVISON *et al.*, 1959) that the metabolism of exogenous labelled cholesterol occurs in the 'grey' matter, while that which has become incorporated in the 'white' matter is stable. A similar stability for cholesterol and other myelin lipids appears likely from the present work for 250 days after the injection of the labelled serine, the 'white' matter is four times more active than the 'grey' when both are expressed in terms of their wet weights.

The present observations on the lipids formed from [3-^{14}C]serine support and extend the earlier demonstration that [4-^{14}C]cholesterol, when injected parenterally into immature rabbits, persists for many months in their brains, spinal cords and sciatic nerves. Since without exception the labelled myelin lipids recovered from these organs show this long persistence, and since the radioactivity is associated with the 'white' rather than with the 'grey' matter, it appears that in the normal animal there is little evidence of any 'turnover' in the lipid constituents of the myelin sheath.

SUMMARY

1. After the parenteral injection of [3-^{14}C]serine into very young rabbits, radioactive cholesterol, sphingomyelin, lecithin, cerebrosides and cephalins can be recovered from their brains, spinal cords and sciatic nerves.

2. In a series of such animals, killed at intervals of from 1 to 192 days after injection, there was little change in the radioactivity of the cholesterol, sphingomyelin and cerebroside fractions recovered from their brains.

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3. In an animal killed after 250 days, the radioactivity of the 'white' matter, when calculated on a weight basis, was about four times that of the 'grey'.

4. The uniform persistence of these three myelin lipids over this long period is consistent with the belief that when once deposited in the myelin sheath at the time of its formation, they undergo little subsequent 'turnover'; the myelin sheath must, therefore, be regarded as one of the more permanent tissue elements.

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A QUANTITATIVE METHOD FOR THE DETERMINATION OF CHLORPROMAZINE IN TISSUES*

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THERE are a number of methods described in the literature for the determination of chlorpromazine (10-(3-dimethylaminopropyl)2-chlorophenothiazine) in biological material (LEACH and CRIMMIN, 1956; SALZMAN and BRODIE, 1956; EIDUSON and WALLACE, 1958; DUBOST and PASCAL, 1955*a*, *b*; CALO, MARIANI and MARIANI MARELLI, 1956). However, none of these is satisfactory because of two major difficulties. In tissues, chlorpromazine is bound very strongly, presumably to proteins (BERGER, 1957), and most of the existing methods do not account for the oxidation products of chlorpromazine and hence measure only chemically unchanged drug—which at least in urine appears to amount to only a small fraction of total excreted drug.

A large proportion of administered chlorpromazine is converted to sulphoxide and is present in this form in urine (SALZMAN and BRODIE, 1956) and possibly also in tissues. Some investigators, however, suspected the existence of additional metabolites either preceding sulphoxide formation or products of further oxidation (SALZMAN and BRODIE, 1956; FRAHM, FRETWURST and SOEHRING, 1956; FELS, KAUFMAN and KARCZMAR, 1958). A chlorpromazine metabolite has been found in the urine of patients who have ingested the drug and has been identified by colour reactions (FORREST and FORREST, 1957; FORREST, FORREST and MASON, 1958). It appears to be a thionium hydroxide derivative since its oxidation level is between that of unchanged chlorpromazine and its sulphoxide (FORREST, FORREST and BERGER, 1958).

We believe our new urinary metabolite to have structure II (Fig. 1), thionium hydroxide, rather than structure IV, a thionol compound, as assumed by de BARRY BARNETT and SMILES (1910), for unsubstituted phenothiazine. However, this does not exclude the possibility of the presence of additional chlorpromazine metabolites in the urine, e.g. one analogous to structure IV. A compound giving the same colour reactions as the urinary metabolite was prepared by irradiating an aqueous chlorpromazine solution with an ultraviolet sunlamp; it contained a small amount of free radicals (III) (FORREST, FORREST and BERGER, 1958).

As far as is known, the method described below encompasses chlorpromazine and its metabolites up to and including the sulphoxide. The sulphone has so far not been encountered by us under physiological conditions in tissues or urine, although recent reports (KAMM, GILLETTE and BRODIE, 1958) suggest that it may be obtained by incubating liver microsomes with cofactors. We have observed shifts in the peaks of ultraviolet absorption spectra resembling the sulphone spectrum when incubating

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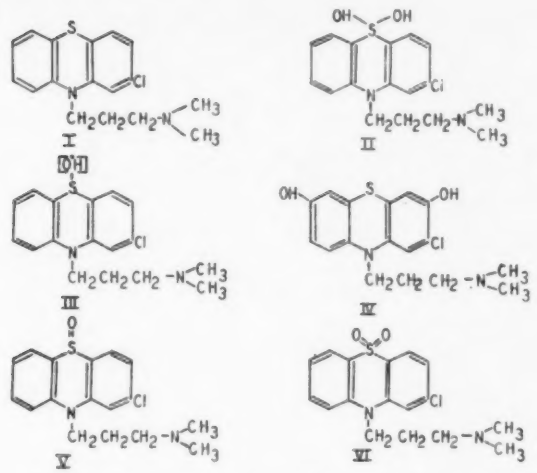

FIG. 1.—Structural formulae of chlorpromazine derivatives.

I Chlorpromazine
II Chlorpromazine thionium hydroxide
III Free radical derived from chlorpromazine thionium hydroxide
IV Chlorpromazine thionol
V Chlorpromazine sulphoxide
VI Chlorpromazine sulphone

chlorpromazine with liver homogenate and cofactors. These shifts, however, may be due to the presence of a mixture of chlorpromazine with the thionium hydroxide compound and sulphoxide.

EXPERIMENTAL AND RESULTS

Colour reactions. The colours obtained with the urine of chlorpromazine treated patients were practically identical with those given by standard solutions of commercial chlorpromazine, sulphoxide, or from the thionium hydroxide prepared by irradiation (Table 1). Solutions of sulphone failed to give any colour reactions.

Method for the determination of chlorpromazine in tissues. The tissues were homogenized, treated with trichloroacetic acid and sodium hydroxide, and subsequently extracted with a mixture of solvents for the removal of chlorpromazine and its metabolites. The solvent phase was treated with a mixture of glacial acetic acid, water, and hydrogen peroxide, which oxidized all chlorpromazine to the sulphoxide level. The sulphoxide was determined by ultraviolet absorption in the Beckman DU spectrophotometer. Standard chlorpromazine sulphoxide samples, either extracted with heptane and subjected to the oxidizing procedure described for tissue, or oxidized by this procedure without heptane extraction, gave the same optical density readings as unoxidized, unextracted chlorpromazine sulphoxide samples. It can thus be assumed that any sulphoxide the tissues may contain prior to oxidation, remains unaffected by the procedure and will not be further oxidized to the sulphone level. Preliminary experiments indicate that some of the tissues, e.g. lungs, contain such preformed sulphoxide. Compared to the chlorpromazine content of the same tissues, however, the amount appears to be rather small. In contrast to the findings with urine, no thionium hydroxide could be detected in the tissues, either before or after extraction.

The ultraviolet absorption spectra of chlorpromazine, its sulphoxide and sulphone are given in Fig. 2.

Procedure. From 0·1 to 1 g of tissue containing the drug were homogenized with 4 ml of water in a glass or Teflon homogenizer. The homogenate was transferred to a 250 ml glass stoppered Erlenmeyer flask and 4 ml of 10% (w/v) trichloroacetic acid followed by 4 ml 10% (w/v) NaOH were added to make the mixture alkaline. Subsequently, 50 ml of *N*-heptane containing 1·5% (v/v) isoamyl

TABLE 1.—COLOUR REACTIONS OF CHLORPROMAZINE AND ITS METABOLITES

Reagents	80 ml 10% H_2SO_4 + 20 ml 5% $FeCl_3$	H_2SO_4, conc.	HCl, conc.
	1 ml	1 ml	1 ml
chlorpromazine 10^{-3}–10^{-5} moles 1 ml	orange-pink	cherry red	pale pink
chlorpromazine thionium hydroxide* 10^{-3}–10^{-5} moles 1 ml	purple	mauve	blue
chlorpromazine sulphoxide 10^{-3}–10^{-5} moles 1 ml	—	deep cherry to purple	deep mauve
chlorpromazine sulphone 10^{-3}–10^{-5} moles 1 ml	—	—	—

* Prepared by irradiation of an aqueous chlorpromazine solution with a UV sunlamp (FORREST, FORREST and BERGER, 1958).

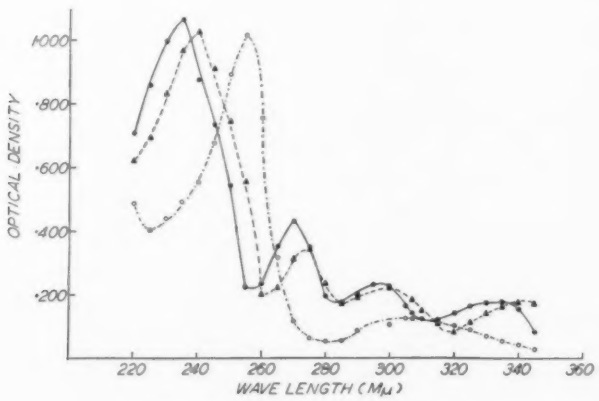

FIG. 2.—UV absorption spectra.

○ - - - - - ○ - - - - - ○ Chlorpromazine

▲ ——— ▲ ——— ▲ Chlorpromazine sulphoxide

● ——— ● ——— ● Chlorpromazine sulphone

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alcohol were added, and the mixture was shaken for 30 min on a Burrell wrist action shaker set at 5. The tissue suspension was centrifuged and the residue was washed once with a small volume of the heptane–isoamyl alcohol extractant. To the solvent phase, 7·5 ml of a mixture containing one vol glacial acetic acid and two vol water were added, and the flask was shaken on the shaker set at 2 for 10 min. Of the acetic acid phase (separated from the solvent phase in a separatory funnel and centrifuged, if cloudy), 3 ml were placed in a test tube with 0·1 ml 30% H_2O_2. The tubes were immersed in a boiling water bath for 2 min, rapidly cooled in cracked ice, and allowed to stand until the temperature had risen to that of the room. The resulting chlorpromazine sulphoxide was determined by measuring the UV absorption in the Beckman spectrophotometer. Readings were taken at wavelengths ranging from 285 to 345 mμ, at 5 to 10 mμ intervals. Chlorpromazine sulphoxide has maxima at 300 and 340 mμ, at which wavelengths the concentration was calculated. Absorption values for tissue from untreated animals subjected to the same procedure were insignificant at these wavelengths, but, nevertheless, mean values of several experiments with control tissues were computed and always subtracted from the optical density readings obtained with the tissues of chlorpromazine treated animals. A standard containing known amounts of chlorpromazine, sulphoxide, or mixtures of both was run through the procedure, and a blank was prepared with acetic acid and hydrogen peroxide, boiled and cooled as above. It is imperative that all experimental conditions be strictly adhered to, since any changes in the concentrations of acetic acid and hydrogen peroxide or in the boiling time may result in shifts and losses of absorption peaks. Furthermore, the shaking time and speed significantly affect the completeness of extraction of chlorpromazine and its sulphoxide from the tissues. With the above specifications optimal extraction was obtained, to the effect that re-extraction of tissues or of the heptane phase showed no further chlorpromazine, or traces only.

Recoveries. Recoveries were determined with both chlorpromazine and the sulphoxide (177–354 μg), or equal mixtures of the two. The substance was added to 1–2 g of tissue homogenized with 4 ml of water, mixed and subjected to the procedure described above. The percentage recoveries were based on values obtained with standard solutions in the absence of tissue. The mean recovery of six experiments with brain tissue was 95% and with liver, 100%. Recoveries with other tissues fell into the above range.

Chlorpromazine levels in liver and brain of rats injected with the drug. Chlorpromazine hydrochloride (SKF Thorazine)* was injected intramuscularly into white Sprague-Dawley rats at a level of 25 mg chlorpromazine per 100 g wt. The average wt of the rats was 300 g. The animals were sacrificed 16–18 hr after injection, and the determination was carried out, whenever possible, on fresh tissues. However, it was found that freezing the tissues did not alter the chlorpromazine level.

The results (Table 2) are based on absorption measurements at both the 300 and 340 mμ peaks with good correlation.

The shaking time for the tissue extraction in the first three experiments (1–3) was 25 min. It was found, however, that there was a lesser possibility of any drug remaining in the tissues when the extraction was continued for 30 min instead of 25, and thus the longer shaking time was adopted. As discussed above, re-extractions of the tissue and heptane phases were carried out in most experiments and showed no measurable amounts of drug.

It is advisable not to measure levels of chlorpromazine below 5 μg, because shifts of absorption maxima or disappearance of peaks may occur. On the other hand, large amounts of the drug, e.g. 3 mg/g of tissue, could be determined without difficulty in suitable dilution. Determinations are being carried out on a number of other tissues with results to be presented in another paper.

The method described above is not directly applicable to determination of urinary chlorpromazine and its oxidative metabolites owing to the presence of thionium hydroxide, as previously discussed, which to date could not be extracted by solvents. A procedure suitable for the determination of total drug and proportions of individual metabolites in urine is being developed and will be presented shortly.

DISCUSSION

A method has been developed for the determination of chlorpromazine in tissue including the oxidative derivatives of the drug. The method incorporates some of the steps described by SALZMAN and BRODIE (1956). However, these workers reported

* We gratefully acknowledge a generous supply of chlorpromazine, its sulphoxide and sulphone from Smith, Kline and French Laboratories.

TABLE 2.—CHLORPROMAZINE LEVELS IN BRAIN AND LIVER OF RATS
(25 mg of drug/100 g wt.)

Exp. no.	Tissue 1 g	mg Chlorpromazine/kg tissue 300 mμ	mg Chlorpromazine/kg tissue 340 mμ
1	Brain	70	65
	Liver	190	190
2	Brain	100	100
3	Brain	80	80
4	Brain	39	35
5	Liver	590	550
6	Liver	730	740
7	Brain	120	110
	Liver	490	480
8	Brain	130	120
	Liver	580	550
Mean brain		90	85
Mean liver		520	500

very poor recoveries of chlorpromazine from tissues, especially brain, from which only 40 per cent of added chlorpromazine could be recovered. We believe that our method measures, except for traces, the total drug present in tissues. As stated above, the thionium hydroxide has not so far been detected in tissues or the tissue residues after extraction by sensitive colour reactions. This does not preclude the presence of traces in a strongly bound form. Conversely, it seems conceivable that thionium hydroxide originally present in tissue underwent secondary oxidation, reduction, dismutation or polymerization. During a mild *in vitro* oxidation of chlorpromazine with hydrogen peroxide to the sulphoxide, the intermediary thionium hydroxide could be fleetingly demonstrated by colour reactions. This is also true of the reverse procedure in which sulphoxide is reduced to chlorpromazine by reducing agents like sodium thiosulphate or zinc and hydrochloric acid.

SUMMARY

(1) A quantitative method for determination of chlorpromazine in tissues is presented. The method is based on the extraction and oxidation of chlorpromazine to the sulphoxide level, at which it is determined by ultraviolet absorption.

(2) Chlorpromazine levels are given for brain and liver of rats injected with the drug.

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NOTICE

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Research and Training Grants

to support the research to increase knowledge of the brain, its functions—normal and abnormal —and sequellae of alterations thereto, by award of project and programme (*long term*) grants.

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Additional information may be procured from:
Director of Research, United Cerebral Palsy Research and Educational Foundation,
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R. E. McCaman and E. Robins: Quantitative biochemical studies of Wallerian degeneration in the peripheral and central nervous systems—II. Twelve enzymes.

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